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STUDIES ON INBREEDING

I. THE EFFECTS IN INBREEDING ON THE GROWTH AND VARIA-
BILITY IN THE BODY WEIGHT OF THE ALBINO RAT

HELEN DEAN KING

The Wistar Institute of Anatomy and Biology

The rapid development of the new science of genetics has opened up many fertile fields of investigation and it has also re-vived interest in the problem of inbreeding which has been dor-mant for many years. Charles Darwin ('75, '78) considered the subject of inbreeding so important that not only did he collect all available data regarding it, but he himself carried on a series of inbreeding experiments that extended over a period of eleven years. Darwin's experiments on plants were followed by those of Crampe ('83), of Huth ('87), and of Retzima-Bos ('93; '94) on various species of mammals. The conclusions reached by each of these investigators can well be stated in the words of Darwin ('78): "The consequences of close interbreeding carried on for too long a time, are, as is generally believed, loss of size, con-stitutional vigor and fertility, sometimes accompanied by a tendency to malformation." Darwin adds, furthermore: "That any evil directly follows from the closest interbreeding has been denied by many persons, but rarely by any practical breeder and never, as far as I know, by one who has largely bred ani-mals which propagate their kind quickly."

On account of the almost universal prejudice against inbreed-ing, or because the results of former work seemed conclusive, the problem of inbreeding was practically ignored by scientists after the publication of Retzima-Bos' results in 1893, and only within the past decade has it again received any serious consid-eration. The recent experiments of Gentry ('05) on swine, of Castle et al. ('06) and of Moenkhaus ('11) on Drosophila, and

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of various stockbreeders on horses and cattle (Chapeaurouge,
'09; Anderson, '11) have shown conclusively that there is no
general physiological law forbidding inbreeding and that the re-
sults obtained depend very largely on the character of the stock
that is inbred. As Chapeaurouge has stated:

Die Erfolge und Miserfolge nächster Inzucht hängen klar und
sicher nicht nur von der Gesundheit und Konstitution der Stammel-
tern und den äusseren Verhältnissen ab, in welchem die Tiere gehalten
werden; sondern auch vor allen Dingen von der richtigen Auswahl der
Tiere zur Weiterzucht, welche nie die allgemeinen Bedingungen des
züchterischen Zweckes gegenüber den speziellen aus dem Auge lassen
darf. Je mehr die Haltung den natürlichen Verhältnissen oder den
Bedürfnissen der Zuchttiere entspricht um so weniger sind üble Folgen
zu befürchten.

There are a number of questions of economic as well as of
scientific import which recent work on inbreeding does not
answer: Does close inbreeding, if carried on for a long period
of time, ever lead to degeneration if only the best animals, from
sound stock, are used for breeding? If degeneration does fol-
low from this kind of mating, does it affect merely the body size,
vigor, and fertility, or does it also influence body form and modify
the structure and action of the central nervous system? If no
evil results appear, can inbreeding be used to improve a race by
combining the best of the dominant characters with any de-
sirable recessive ones that may appear? Finally, does inbreeding
change the normal sex ratio, as Düsing ('84) and others have
maintained? Taking advantage of the opportunity which the
animal colony of The Wistar Institute of Anatomy and Biology
afforded for carrying on an investigation that might answer
some of the above questions, a series of inbreeding experi-
ments on the albino rat was started in the spring of 1909.
As the work, although still in progress, has already extended
over a period of eight years, it seems advisable that some of the
results obtained should be published, since they lead to rather
definite conclusions. The present paper, the first of a series,
gives a detailed account of the investigation, with an analysis
of the effects of close inbreeding on the growth and variability

in the body weight of rats belonging to fifteen inbred generations; subsequent papers will deal with the effects of inbreeding on fertility, on constitutional vigor and on the sex ratio. To avoid needless repetition, a complete bibliography will be given in the final paper.

I wish to express my great obligations to the Director of The Wistar Institute, Dr. M. J. Greenman, for placing at my disposal ample facilities for carrying on the investigation and for his unfailing interest in the problem. I am much indebted, also, to Dr. H. H. Donaldson for advice and criticism that have been most helpful when difficult situations have arisen at various stages of the work.

1. MATERIAL AND OUTLINE OF THE EXPERIMENT

The albino rat (Mus norvegicus albinus) is a domesticated variety that is well adapted to confinement and is easily cared for. It breeds throughout the year, producing litters of relatively large size, and under proper environmental conditions from three to four generations can be obtained in a single year. This combination of characteristics makes the rat very useful as a laboratory mammal, and exceptionally favorable material for a study of the problem of inbreeding.

The experiments were started with a litter containing four rats, two males and two females, which was born on the 10th of May, 1909. This litter was selected from a number of new-born litters in the general stock colony merely because it happened to contain the number of individuals required for starting the investigation, and not because of the ancestry, the size, or the vigor of the individuals. One of the females in the litter was called 'A,' and her descendants form the 'A series' of inbreds; the other female was designated as 'B,' and her descendants form the 'B series' of inbreds.

The plan of the experiment required that females A and B, as well as all of the females subsequently used for breeding, should be mated twice with a brother from the same litter and then twice with an unrelated stock male. Since the mating of

brother and sister from the same litter is the closest form of
inbreeding possible in mammals, no other form of mating has
ever been used to obtain inbred litters. In every generation
all females used for breeding have belonged to inbred litters;
none of them have ever been taken from 'half-inbred' litters
obtained by the mating of inbred females with stock males.
In the early part of the experiment the number of breeding
animals was, of necessity, small. In every generation after the
sixth about twenty females from each series were used for
breeding, so that approximately 1000 young were obtained in
each generation.

All four of the rats used in starting the experiment were
killed when they were no longer wanted for breeding purposes.
Each rat was weighed, measured, and carefully dissected. When
the various records were compared with the norms for the albino
rat (Donaldson, '15) it was found that all of the rats were under
the average body weight for their age, but that they were nor-
mal in all other respects as far as could be determined by the
usual methods of laboratory procedure. The fact that these
individuals were sound, healthy animals and normal in all
essential respects is a point on which I wish to place special
emphasis in order to forestall the possible criticism that the re-
sults obtained in this work were due to the use of an exceptional
strain of rats.

In the earlier generations the inbred rats exhibited all of the
defects which are popularly supposed to appear in any closely
inbred stock. Many females in both series were sterile, and
those that did breed usually produced only one or two litters
which were generally of small size. A considerable proportion
of the rats were dwarfed, or stunted in their growth, and many
of them developed malformations, particularly deformed teeth.
The animals showed, also, a steady decline in vitality in suc-
ceeding generations and usually died at a relatively early age.
If the experiments had been discontinued at this point the re-
sults would have been a confirmation of the conclusion reached
by Darwin and by several other investigators, that inbreeding
invariably leads to sterility and to physical degeneration.

Fortunately for this work, many rats in the general stock colony, in which there was no inbreeding, exhibited the same characteristics as the rats belonging to the inbred strain. It was evident, therefore, that the unfavorable condition of the animals could not justly be attributed to inbreeding alone. On investigation it was found that all of the rats were suffering from malnutrition due to the character of the food that they received. At the time that these experiments were begun the rat was used as a laboratory mammal in only a few of the larger research institutions in the country, and little was known of the environmental and nutritive conditions best suited to its needs. Following the plan of feeding in general use in other animal colonies, the rats were fed chiefly on bread soaked in milk and on corn; meat and vegetables being given only once each week: Such a diet does not furnish the proportion of food elements that the rat requires if it is to be kept in good physical condition: there is too much starch, too little protein. In the spring of 1911 a radical change was made in the rats' food. Milk and fresh bread were eliminated from the diet and 'scrap' food, consisting of carefully sorted table refuse, was fed once each day; cobcorn being kept in the cages as an extra ration. Such a diet has proved to be a most satisfactory one, and it has been used continually up to the present time, except that dog biscuit has been substituted for cobcorn as extra food supply. This change was made last year following the loss of a considerable number of animals through intestinal disturbance caused by the eating of fermented corn.

A very marked improvement in the general condition of all of the rats in the colony was noted very soon after the diet was changed. The animals gained in size and in weight, sterility almost disappeared, and the average number of young in the litters was increased. From this time on malformations were no longer common, and not a single instance of deformed teeth has been discovered in the thousands of animals that have been bred in the colony during the past five years. Simply by a change in the food characteristics said to typify the 'dire effects of inbreeding' were eliminated, and up to the present time they

have not reappeared, although the rats have been carried through twenty-eight generations of brother and sister matings.

In the inbred colony, up to the sixth generation, very little selection of breeding animals was possible; any females that would breed at all were used to continue the series. The change in food was made at the time that the animals of the fourth inbred generation were reaching maturity. In the course of the two following generations the effects of malnutrition gradually disappeared, and in the sixth generation most of the rats were of normal size and relatively large litters were being produced. After this time large and vigorous animals were available for breeding purposes, and it became possible to make a careful selection of the breeding stock.

From the seventh generation on the selection of the individuals which were to serve as progenitors of the succeeding generation was always made among the newborn young, as the sexes can readily be distinguished at this time (Jackson, '12). In the A series of inbreds, which is called the 'male line,' all litters containing an excess of female young were always discarded; in the B series, the 'female line,' litters with an excess of male young were never reared. Unless the individuals in the litter were of normal size and vigorous at birth they were killed at once. The young which were retained remained with their mother until they were one month old, when they were again carefully examined, and if they did not come up to the norms for stock animals of like age they were discarded. If the young rats fulfilled all requirements as to body weight and vigor they were returned to the cage to be reared as possible breeding stock. This rigid selection left in each generation, as a rule, at least three times the number of animals that were required for breeding. When the rats become sexually mature, at about three months of age, they were again inspected, and any that were below normal in any way were rejected. Generally only one female of a litter, the first to breed, was taken to continue the line. If, however, the individuals were unusually large and vigorous, two, very rarely three, breeding females were taken from the same litter.

Since the change in the food in 1911 and the removal of the colony to new quarters in 1913, the environmental conditions under which the rats were reared have been as uniform as it was possible to make them. All inbred rats, and also the stock animals used for controls, have been subjected to the same conditions of light, of temperature, and of nutrition, and they have been cared for in a similar way. Any differences between the two inbred series, or between inbred and stock animals must, therefore, be ascribed to causes inherent in the individuals; they cannot be attributed to the varying action of environment or nutrition.

2. THE GROWTH IN BODY WEIGHT OF INBRED RATS

In view of the results that earlier investigators (Crampe, Ritzema-Bos) obtained in their inbreeding experiments with rats, little attention was paid to the fact that the body weights of the animals in the earlier inbred generations were considerably less than the norms for stock albino rats of like age. When the individuals of the sixth inbred generation became mature, it was noted that many of them were much larger than stock animals of the same age. This fact was so at variance with the generally accepted belief regarding the effects of close inbreeding on body size that it seemed desirable to make a study of the weight increase with age of individuals in the later generations of the two inbred series.

From the seventh generation on from three to five litters of each inbred series were weighed, first when the animals were thirteen days old, again when they were weaned at thirty days of age, and thereafter at intervals of one month until they were fifteen months old. At the thirteen- and thirty-day periods animals of the same sex were weighed together and the average body weight for the group recorded, as at these ages individual differences in body weight are, as a rule, too small to make separate weighings necessary; at all other ages individual records were taken.

The litters that were used for a study of growth in body weight were all selected at birth on the same basis as the litters

that were to be reared for breeding purposes. There was no culling of the less desirable individuals, however, and all members of every litter were reared and weighed at the ages noted. When the animals became mature the largest and most vigorous pair in each litter was usually used for breeding.

As the growth of very young rats depends largely on the amount of nourishment that they receive from their mother, litters of medium size, containing from five to eight young, were, as a rule, those selected for weighing. Such litters, moreover, represent the general run of individuals in a colony more fairly than do very large or very small litters in which animals with extreme body weights are often found. It was not always possible to weigh adult animals at exactly the ages designated in the various tables, but the weighing of a litter was omitted if it could not be taken within one week of the time specified.

In weighing experiments of this kind there is always an unavoidable error due to the presence of a greater or a less amount of undigested food in the alimentary tract. To obviate this source of error as far as possible the rats were weighed in the morning before they had received their daily food ration. Animals that were obviously ill and females known to be pregnant were never weighed, while the weight of suckling mothers was not recorded if it was below the previous record. In the rat pregnancy cannot be detected with certainty until about the thirteenth day, so undoubtedly many gravid females were weighed unknowingly during the course of this investigation. The increase in the body weight of a female as the result of pregnancy cannot be very great up to the thirteenth day, however, since Stotsenburg ('15) has shown that the weight of a fetus at this time is only 0.04 grams. Errors in the records due to the inclusion of the weights of pregnant females were doubtless balanced by the weights for animals that were in early stages of pneumonia when there was no external evidence of the disease.

The present paper gives data showing the weight increase with age in 333 males and in 306 females belonging to the first fifteen generations of the inbred group. Altogether these generations comprised a total of 1601 litters, containing 11,657

individuals, of which the majority were the progeny of brother and sister matings, the others were the offspring of inbred females and stock males. The number of animals for which weight records were taken is, it is hoped, large enough to be fairly representative of the inbred colony as a whole and to give results that have some statistical value.

No animals belonging to the first six inbred generations were weighed at regular intervals, but fortunately a series of weight records is available that will give some idea of the size of these rats after they became adult. In order to ascertain the effects of close inbreeding on the weight of the central nervous system, one of my colleagues, Dr. S. Hatai, made careful autopsies of the four rats used in starting the experiment and he also examined a large number of their descendants. From the record cards for these animals I was able to obtain the body weights of a considerable number of rats belonging to the first six generations of the two inbred series. The body weight data for only the best of the animals reared during this period were used in the present case; records for all individuals that were noted as having deformed teeth or other malformations were excluded, as well as the records for those animals that were obviously runts.

Table 1 shows the body weight records for 92 males and for 73 females belonging in the first six generations of the A series of inbreds: table 2 gives similar data for 85 males and for 64 females of the B series. Only one record for each individual was obtained, i.e., the body weight at the time that the animal was killed.

. The 'mean age in days,' as given in the first column of table 1 and of table 2, shows the median points in thirty periods that correspond to the ages at which the rats of the later inbred generations were weighed. For example, under the mean age '151 days' are grouped the body weights of all of the animals that were killed when they were from 136 to 165 days of age. As no records were taken of animals that were less than 105 days old the first age group given is that for 120 days.

TABLE 1

Showing the increase in the weight of the body with age for rats belonging in the first six generations of the A series of inbreds

MEAN AGE	MALES				FEMALES			
	Body weight in grams			Number of individuals	Body weight in grams			Number of individuals
	Average	Highest	Lowest		Average	Highest	Lowest	
days								
120	200.1	252	134	11	142.8	193	106	13
151	188.2	267	133	24	127.5	165	103	15
182	218.2	317	135	17	132.2	199	105	5
212	183.6	227	138	6	123.7	153	102	8
243	239.8	293	182	11	177.5	178	177	2
273	225.0	292	155	11	146.4	218	112	5
304	246.2	273	203	5	137.6	143	128	3
334	274.0	312	225	3	189.0	225	162	5
365		310		1*	164.8	215	143	5
395	273.0	294	269	3	177.1	191	151	6
425						166		1*
455					164.8	180	152	5
				92				73

* Record not used in constructing graph.

TABLE 2

Showing the increase in the weight of the body with age for rats belonging in the first six generations of the B series of inbreds

MEAN AGE	MALES				FEMALES			
	Body weight in grams			Number of individuals	Body weight in grams			Number of individuals
	Average	Highest	Lowest		Average	Highest	Lowest	
days								
120	205.8	266	142	24	149.7	170	136	4
151	210.6	257	141	16	149.4	211	113	11
182	241.6	345	146	15	136.0	185	109	5
212	235.7	315	157	12	119.2	173	100	4
243	270.5	304	238	4	169.7	189	149	4
273	215.6	241	185	6	161.0	193	131	4
304	233.7	287	174	4	161.7	189	138	12
334		249		1*	191.6	213	161	6
365	223.0	257	202	3	163.6	185	144	5
395					167.8	193	139	5
425								
455					172.2	213	145	4
				85				64

* Record not used in constructing graph.

A comparison of the corresponding data for the individuals of the two series of inbreds is best made through the graphs in figure 1 constructed from the data for the average body weights at different ages as given in table 1 and in table 2.

In figure 1 the graphs for the body growth of the males are considerably higher than those for the females, since the male rat, after reaching maturity, is normally a much heavier animal than the female of like age. Considering that only one record

Fig. 1 Graphs showing the increase in the weight of the body with age for males and females belonging in the first six generations of the two series of inbred rats (data in table 1 and in table 2).

was taken for each animal the corresponding graphs for the two series, especially those for the females, run remarkably close together. It is evident, therefore, that there was no significant difference between the two series as regards the growth of the individuals during the first six generations of inbreeding.

Table 3 gives the body weight data for all of the individuals of the first six inbred generations for which records were taken (a combination of the data in table 1 and in table 2).
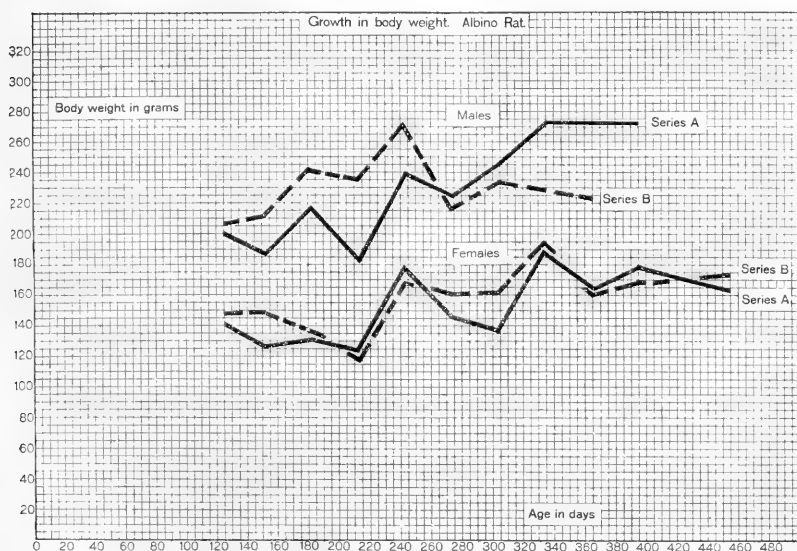
Showing the increase in the weight of the body with age for rats belonging in the first six generations of the inbred series. A combination of the data in table 1 and in table 2

MEAN AGE	MALES				FEMALES			
	Body weight in grams			Number of individuals	Body weight in grams			Number of individuals
	Average	Highest	Lowest		Average	Highest	Lowest	
days								
120	204.0	266	134	35	144.4	193	106	17
151	197.2	267	133	40	136.6	211	103	26
182	229.1	317	135	32	139.1	199	105	10
212	218.3	315	138	18	122.2	173	100	12
243	248.0	304	182	15	172.3	189	149	6
273	221.7	292	155	17	152.8	218	112	9
304	240.6	287	174	9	156.9	189	128	15
334	267.7	312	225	4	190.4	225	161	11
365	244.7	310	202	4	164.2	215	143	10
395	273.0	294	269	3	172.9	193	139	11
425						166		1*
455					168.1	213	145	9
				177				137

* Record not used in constructing graph.

A comparison of the data in table 3 with body weight data for a series of stock albino rats reared as controls for the inbred series after the change to 'scrap' diet had been made (table 13) will show to what extent malnutrition decreased the body size of the individuals in the first six inbred generations. Graphically this result is shown in figure 11 and in figure 12 (compare graph D with graph A and graph C).

Body weight data were taken, at the intervals stated, for 99 males and for 76 females belonging in the seventh to the fifteenth generation of the A series of inbreds. The average body weights of the males at different age periods are shown, by generations, in table 4: corresponding data for the females are given in table 5.

Data for 57 males and for 93 females belonging in the seventh to the fifteenth generations of the B series of inbreds are shown according to generations in table 6 and in table 7.

Showing, by generations, the average body weight at different ages of 99 males belonging in the seventh to the fifteenth generations of the A series of inbred rats

AGE	GENERATIONS								
	7	8	9	10	11	12	13	14	15
days	grams	grams	grams	grams	grams	grams	grams	grams	grams
13	18	18	18	18	16	19	19	18	18
30	43	45	46	41	44	44	49	47	42
60	150	77	134	129	102	127	134	123	123
90	201	151	199	214	177	184	186	193	189
120	242	196	249	264	227	229	244	233	204
151	274	252	287	289	257	249	273	259	235
182	306	286	308	315	279	273	291	273	256
212	312	315	330	299	288	293	311	283	266
243	329	341	342	318	302	295	317	293	286
273	355	352	364	305	321	312	327	304	300
304	357	345	334	310	314	319	333	302	303
334	368	368	376	305	327	316	339	307	307
365	374	377	399	300	336	324	353	317	315
395	375	370	386	307	360	318	343	318	312
425	412	384	404	307	354	334	355	330	321
455	424	383	403	324	343	339	344	322	320
Number of rats weighed..........	6	6	9	14	12	10	15	15	12

Showing, by generations, the average body weight at different ages of 76 females belonging in the seventh to the fifteenth generations of the A series of inbred rats

AGE	GENERATIONS								
	7	8	9	10	11	12	13	14	15
days	grams	grams	grams	grams	grams	grams	grams	grams	grams
13	16	15	16	15	15	18	17	17	18
30	40	37	42	37	41	41	45	45	40
60	126	73	116	107	90	102	110	108	105
90	160	117	156	184	139	145	157	162	144
120	172	131	181	184	181	168	172	171	163
151	190	163	205	189	182	187	192	185	183
182	190	182	217	208	189	195	196	204	203
212	187	193	211	193	184	218	215	209	211
243	189	200	223	202	200	203	216	214	218
273	187	219	229	223	192	226	218	217	222
304		221	221	236	209	239	221	222	222
334		217	220	242	222	244	218	223	227
365		252	256	256	223	238	220	226	226
395		232		253	226	253	227	228	224
425		252		244	260*	268*	230	226	219
455		241		257	257*	243	224	219	217
Number of rats weighed..........	4	9	7	8	10	8	9	10	11

* One record only.

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TABLE 6

Showing, by generations, the average body weight at different ages of 57 males belonging in the seventh to the fifteenth generations of the B series of inbred rats

AGE	GENERATIONS								
	7	8	9	10	11	12	13	14	15
days	grams	grams	grams	grams	grams	grams	grams	grams	grams
13	21	21	20	18	19	19	19	20	20
30	49	49	53	49	40	47	50	52	49
60	170	158	140	172	149	140	144	132	145
90	244	197	211	217	220	212	193	210	201
120	277	280	253	265	262	240	225	255	233
151	322	293	288	291	300	264	274	279	267
182	344	328	340	334	330	278	283	300	288
212	376	331	360	340	353	290	297	305	303
243	372	348	367	336	368	286	303	302	308
273	454*	356	371*	316	367	,306	309	311	311
304	477*	361	380*	335	408	313	313	322	311
334		361	372*		381	337	303*	330	321
365		364	345*	333	373	328	326*	362	336
395		369		365*	365	336*	334*	351	339*
425		365*			353			357*	339*
455					347*			343*	330*
Number of rats weighed.........	3	7	3	5	9	8	6	7	9

* One record only.

TABLE 7

Showing, by generations, the average body weight at different ages of 93 females belonging in the seventh to the fifteenth generations of the B series of inbred rats

AGE	GENERATIONS								
	7	8	9	10	11	12	13	14	15
days	grams	grams	grams	grams	grams	grams	grams	grams	grams
13	18	18	17	17	18	18	18	18	18
30	45	40	50	48	39	39	46	48	45
60	135	119	122	125	113	120	104	104	107
90	187	168	168	170	165	149	147	161	151
120	187	181	180	174	182	167	169	183	172
151	204	200	200	199	191	186	195	196	191
182	213	196	205	208	200	195	196	214	202
212	216	213	199	217	202	204	204	210	206
243	208	216	211	217	225	212	214	216	204
273	243	196*	218	212*	236	208	220	219	214
304	246	201*	217	239	243	207	225	223	217
334	235	204*	239		255	211	243	237	224
365	249	210*	242	262*	249	215	238	241	229
395	301*		241	275*	241	212	226	239	229
425	323*			261*	238	207		239	234
455	317*			280*	244			231	235
Number of rats weighed.........	7	11	5	11	12	10	9	12	16

* One record only.

Tables 4 to 7 are inserted chiefly for reference, although they bring out two important facts more clearly, perhaps, than do any of the other tables. The data, as given in these tables, show that rats belonging to the earlier generations of the inbred series did not live as long, as a rule, as did the individuals in the later generations. This was, particularly noticeable in the individuals of the B series. Up to the twelfth generation only three rats in the B series (two females and one male) lived to the age of 455 days; in subsequent generations many individuals lived for the entire weighing period of fifteen months, and some of them were kept until they were nearly two years old. One who believes with Crampe and Ritzema-Bos that continued inbreeding necessarily lessens vitality and so shortens the life of the individual meets here with the seemingly paradoxical fact that the animals that belonged to the later inbred generations outlived those that belonged to the earlier generations. In this experiment the use of only the most vigorous animals for breeding purposes has seemingly overcome any tendency that inbreeding might have to shorten the life of the individuals.

The second point of interest brought out by tables 4 to 7 is that in each generation of the two inbred series the average body weight of the males exceeded that of the females at every age for which records were taken. At birth the male albino rat is slightly heavier than the female, whether the animals belong to a stock or to an inbred strain (King, '15 b). Data for the growth in body weight of the albino rat, as recorded by Donaldson ('06), show that as early as the seventh day after birth the growth of the female is more vigorous than that of the male, and that the female is, as a rule, a relatively heavier animal than the male up to about fifty-five days of age. Ferry's ('13) growth data for the albino rat (Donaldson, '15; table 65) confirm Donaldson's findings. In Jackson's ('13) data for the albino rat, "the excess of average weight was invariably in favor of the male at birth, and also in the majority of cases at all succeeding ages;" while the records obtained by Hoskins ('16) show that the albino female is a heavier animal than the male only at the age of about six weeks. In a series of stock albino

rats reared as controls for the inbred series (King, '15 a) the majority of the males exceeded the females in body weight at each weighing period. As no records were taken when the animals were just six weeks old, the relative size of the two sexes at this age was not determined.

At thirteen days of age, as tables 4 to 7 show, the average body weight of the inbred males was only one gram more than that of the females. Such a slight difference as this would be negligible, considering the possible error in the weighings due to the varying amount of food in the alimentary tract, save for the fact that it is found in every generation group. In some litters the females, were, on the average, heavier than the males at thirteen and also at thirty days of age, and in a few instances the weight of the largest female surpassed that of the smallest male even when the animals were sixty days old. Although the albino female, whether stock or inbred, has a relatively smaller birth weight than the male, she soon comes to have a body weight that is very nearly equal to that of the males in all cases, and often exceeds it. Even though the absolute body weights are less at any given age, therefore, the female grows more vigorously than the male during the first few weeks of postnatal life. An early acceleration in the growth of the female also occurs in the guinea-pig (Minot, '91; Castle, '16), and it finds a parallel in man, as Donaldson ('12) has pointed out, since during a certain phase of development, when children are from thirteen to sixteen years old, the average weight of girls is greater than that of boys; while at all other ages boys, as a rule, are the heavier.

The relative growth in body weight of males and of females in corresponding generations of the two inbred series, or in succeeding generations of the same series, can be determined by referring to the data in tables 4 to 7. For a comparative study of the body growth of the individuals in the two series it seemed advisable to combine the data for three succeeding generations. Data for the A series of inbreds are given in table 8.

The growth graphs shown in figure 2 were constructed from the data for the males of the A series, as given in table 1 and

TABLE 8

Showing the average body weights at different ages of inbred rats of the A series separated into three groups according to the generation to which the individuals belonged

AGE	MALES			FEMALES		
	Generations 7–9	Generations 10–12	Generations 13–15	Generations 7–9	Generations 10–12	Generations 13–15
days	grams	grams	grams	grams	grams	grams
13	18.1	17.5	18.4	15.5	16.0	17.5
30	49.4	43.4	43.6	42.5	40.0	43.2
60	122.3	118.3	127.0	98.9	98.5	107.6
90	186.0	193.2	188.9	137.5	157.9	153.6
120	230.6	242.5	228.1	160.5	178.5	168.3
151	269.5	267.9	256.9	181.5	191.0	186.2
182	304.3	290.4	276.7	192.2	195.3	201.9
212	320.2	293.2	287.1	189.8	196.2	211.2
243	337.1	305.9	298.3	205.5	201.7	216.0
273	357.4	312.5	307.7	214.7	215.1	219.4
304	349.5	314.5	313.7	221.1	219.4	221.7
334	369.0	315.4	318.4	227.1	234.9	223.5
365	379.2	318.1	327.1	252.2	235.2	224.7
395	375.6	323.6	323.6	232.0	239.5	226.4
425	399.4	327.0	332.0	252.5	254.0	223.8
455	403.5	336.0	333.2	241.0*	251.6	219.0

* One record only.

in table 8. In this, as in some of the other figures, the space between the graphs was slightly widened, where properly the lines would run very close together or overlap, in order that the course of each graph might be clearly followed.

Figure 2 shows that the body growth of the males in the three generation groups of the A series of inbreds progressed at about the same average rate until the animals were 150 days of age, as is indicated by the position of graphs A to C. At this point there was a marked acceleration in the growth of the males belonging to the group comprising the seventh to the ninth generation (graph A) which continued until the end of the weighing period. Graph D, representing the growth of the males of the A series during the first six generations, begins at the 120-day period, as no younger rats belonging to these generations were weighed. It seems almost incredible that this graph can

represent the adult body size of the progenitors of the animals whose growth is indicated by graph A, since rarely do we find a group of mammals having in the adult state a body size so much greater than that of its immediate ancestors.
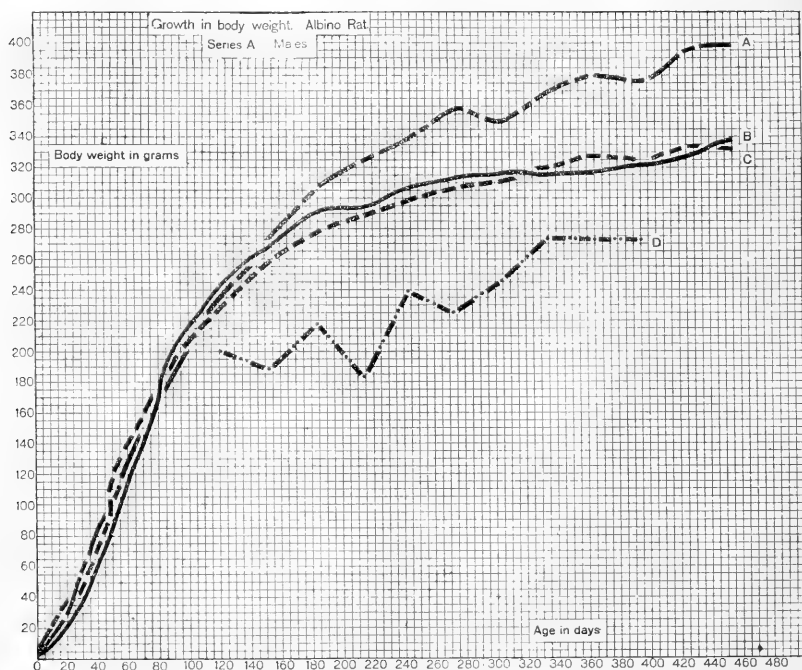

Fig. 2 Graphs showing the increase in the weight of the body with age for males belonging to various generation groups of the A series of inbreds (data in table 1 and in table 8). A, graph for males of the seventh to the ninth generations inclusive; B, graph for the males of the tenth to the twelfth generation inclusive; C, graph for the males of the thirteenth to the fifteenth generations inclusive; D, graph for the males of the first six inbred generations.

Graphs showing the growth in body weight of females belonging to the three generation groups of the A series are shown in figure 3 (data in table 1 and in table 8).

The females of the first six generations of the A series were very much smaller than the females of the later generations at every age for which records were taken, as the position of graph D in figure 3 clearly shows. There was practically no difference in the rate or in the extent of the body growth in the groups

comprising the seventh to the fifteenth generations, as graphs A, B and C cross and recross each other at various points and run as close together as would any set of graphs constructed from the data for different series of individuals.

Data for the growth in body weight of the-individuals of the B series, arranged in groups of three generations each, are given in table 9.
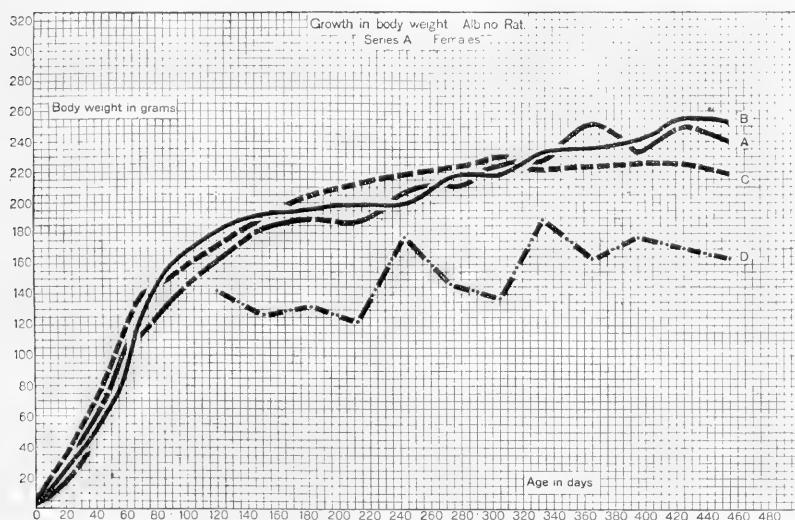
Fig. 3 Graphs showing the increase in the weight of the body with age for females belonging to various generation groups of the A series of inbreds (data in table 1 and in table 8: lettering as in figure 2).

From the average body weights at different ages of the males of the B series of inbreds, as given in table 2 and in table 9, the graphs in figure 4 have been constructed.

In the B series, from the first weighing until the last, there was a marked difference in the body weights of the males in the four generation groups, since all of the graphs in figure 4 are distinctly separated except at one point (365-day period). Graph A runs higher than any of the other graphs from the beginning until the end of its course, thus indicating that in this series of inbreds also the males of the seventh to the ninth generation were heavier animals than the males in the later generation groups. Males in the first six generations of the B series

TABLE 9

Showing the average body weights at different ages of inbred rats of the B series separated into three groups according to the generation to which the individuals belonged

AGE	MALES			FEMALES		
	Generations 7–9	Generations 10–12	Generations 13–15	Generations 7–9	Generations 10–12	Generations 13–15
days	grams	grams	grams	grams	grams	grams
13	21.0	19.1	19.8	17.9	17.7	18.2
30	50.0	47.2	50.0	43.8	44.8	46.2
60	156.9	151.4	140.7	120.3	119.6	105.4
90	218.9	213.4	201.6	172.8	163.3	153.6
120	273.6	259.7	237.6	182.8	175.9	174.6
151	299.0	284.3	271.6	201.3	190.4	193.5
182	335.3	309.6	289.7	205.3	200.6	205.4
212	347.5	323.4	301.7	210.4	209.5	206.7
243	358.3	321.4	304.9	211.6	217.7	210.5
273	372.2	330.4	310.2	222.2	217.1	217.1
304	380.5	353.2	316.5	225.6	222.4	221.3
334	363.1	359.0	323.1	230.6	228.6	230.8
365	360.4	342.4	344.2	238.4	229.3	234.3
395	369.0	357.7	342.2	261.3	236.4	232.6
425	365.0*	353.0	348.0	323.0*	230.4	236.4
455		347.0*	331.5	317.0*	256.3	233.2

* One record only.

TABLE 10

Showing the average body weights at different ages of inbred rats of the combined series (A, B) separated into three groups according to the generation to which the individuals belonged

AGE	MALES			FEMALES		
	Generations 7–9	Generations 10–12	Generations 13–15	Generations 7–9	Generations 10–12	Generations 13–15
days	grams	grams	grams	grams	grams	grams
13	19.2	18.1	18.9	16.8	16.9	17.8
30	49.6	44.9	47.6	43.0	42.8	45.1
60	135.5	131.8	131.7	112.7	110.7	106.4
90	195.6	200.9	193.1	154.1	160.7	153.6
120	248.6	250.5	231.4	172.1	177.2	171.8
151	281.1	274.3	262.2	191.2	190.7	190.2
182	315.6	298.0	279.5	199.2	198.5	203.5
212	328.7	305.7	291.6	203.4	202.2	208.3
243	343.9	311.4	300.0	208.6	209.4	213.3
273	361.6	316.1	308.5	217.4	215.8	218.2
304	359.8	322.2	314.2	222.8	220.8	221.5
334	367.3	324.1	319.3	228.7	232.8	226.6
365	374.0	325.5	329.2	243.7	232.4	228.6
395	374.4	331.4	325.3	246.6	238.3	229.0
425	396.2	330.5	333.4	276.0	240.8	229.6
455	403.5	336.7	333.0	279.0	253.3	224.6

were quite as inferior to their descendants in body size as were
the males in the corresponding generations of the A series
(graph D).

The growth in body weight of various groups of females be-
longing in the B series is shown graphically in figure 5 (data in
table 2 and in table 9).

Graphs A to C in figure 5 were drawn so that the lines
are distinct. These graphs should lie very close together and

Fig. 4   Graphs showing the increase in the weight of the body with age for
males belonging to various generation groups of the B series of inbreds (data in
table 2 and in table 9: lettering as in figure 2).

overlap in many places, since the data in table 9 shows that the
actual differences between the corresponding body weights of
the various groups are very small. The position of graph D
indicates that adult females of the first six generations were
very much smaller animals than their descendants of like age,
as was the case in the A series also.

The data given in table 8 and in table 9 have been combined
in table 10.

Figure 6 shows graphs for the weight increase with age in all
of the inbred males for which weight records were taken (data in
table 3 and in table 10).

As the position of the graphs in figure 6 show, males of the
seventh to the ninth inbred generations (graph A) were animals
of unusually large size and they were considerably heavier,
after reaching maturity, than their own progeny of like age. At
the 365 day period the space between graph A and graph B in-
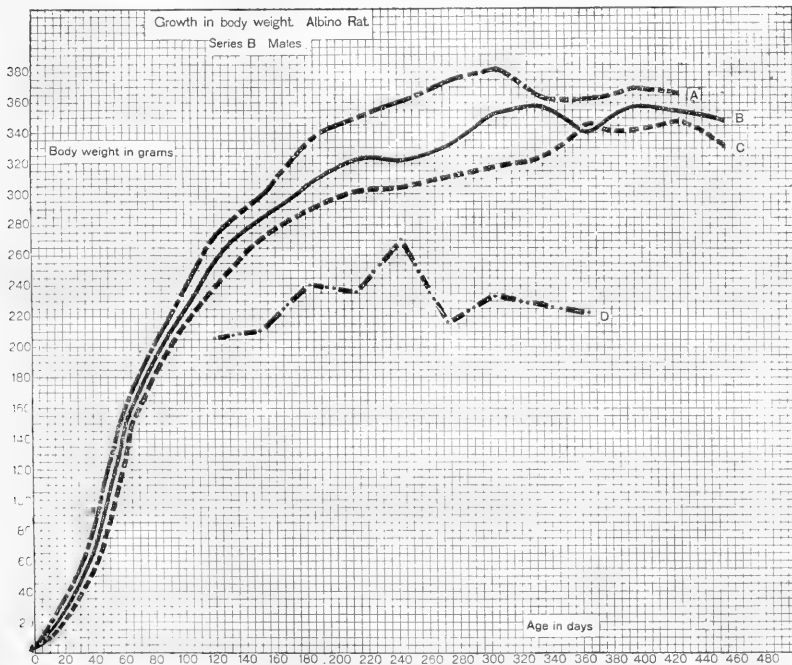dicates a difference of about 50 grams in the average body

Fig. 5   Graphs showing the increase in the weight of the body with age for
females belonging to various generation groups of the B series of inbreds (data
in table 2 and in table 9: lettering as in figure 2).

weights of the two groups of animals.   From the tenth genera-
tion on the course of growth in body weight was practically the
same in all inbred males, as is shown by the fact that graph B
and graph C run very close together throughout their entire
length.

Figure 7 shows graphs for the body weight increase in the
series of inbred females (data in table 3 and in table 10).

The relative position of the graphs in figure 7 show that,
with the exception of the animals in the first six inbred genera-

Fig. 6 Graphs showing the increase in the weight of the body with age for males belonging to various generation groups of the two series combined (A, B). Data in table 3 and in table 10: lettering as in figure 2.
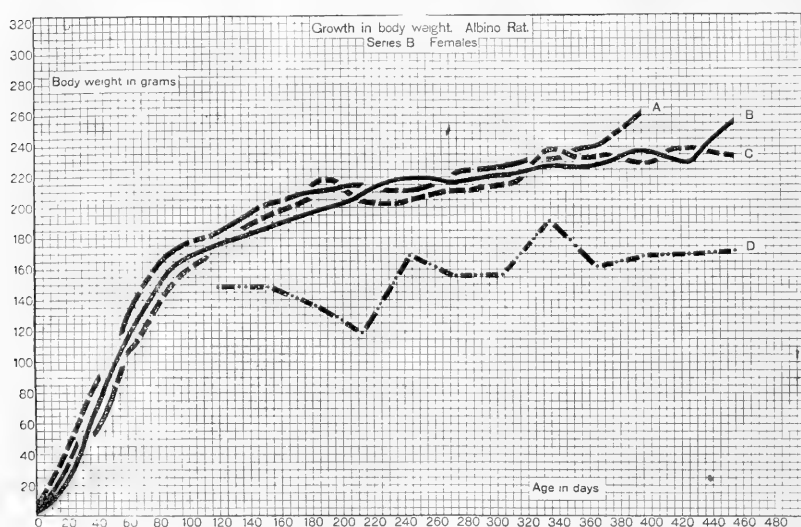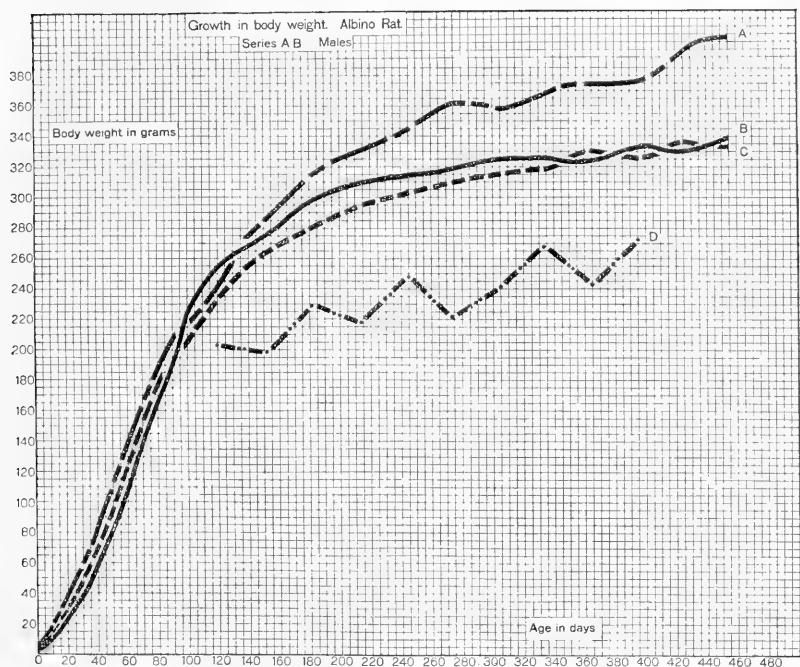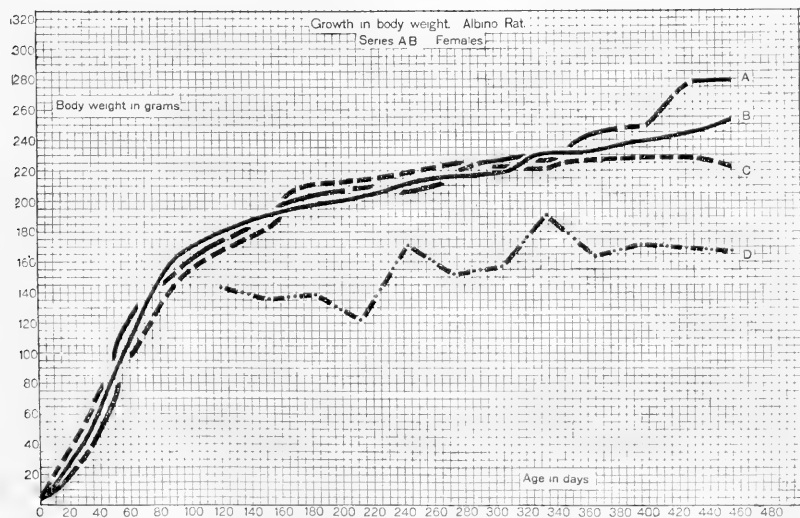

Fig. 7 Graphs showing the increase in the weight of the body with age for females belonging to various generation groups of the two series combined (A, B). Data in table 3 and in table 10: lettering as in figure 2.

tions (graph D), the body increase with age was about the same in all the various generation groups of inbred females up to the time that the animals were one year old. After this age the females in the group comprising the seventh to the ninth generations were somewhat heavier than the other females, but their excess in body weight was very much less than that in the corresponding groups of males (fig. 6).

On referring to the data in table 4 to table 7 it is found that in both series of inbreds the average body weights of the animals in the seventh, eighth and ninth generations were greater than those of animals in the tenth and subsequent generations. In the seventh generation, particularly, females as well as males were exceptionally heavy animals at all ages for which records were taken. The largest males yet obtained belonged in the seventh generation of the A series of inbreds; these rats weighed at their maximum 460, 482, and 511 grams respectively. The largest female in the series was a member of the seventh generation of the B series of inbreds, and she weighed 323 grams when she was 425 days old. The probable cause for the unusual weight of the animals in the seventh to the ninth inbred generations will be considered later.

Data showing the range in variation and the averages for the body weights at different ages of the weighed individuals in the A series of inbreds (seventh to fifteenth generations only) are given in table 11. Similar data for the rats of the B series are shown in table 12.

The graphs in figure 8 were drawn to show the relative growth in body weight of the males belonging to the two inbred series (data in table 11 and in table 12).

The males of the B series of inbreds had a heavier average body weight than the males of the A series up to the last weighing period (455 days), as the position of the graphs in figure 8 show. The crossing of the graphs at the end has no significance, since the largest males of the B series died before the final weighing.

Graphs showing the growth in body weight of the females in the two inbred series are shown in figure 9: the data for these graphs are given in table 11 and in table 12.

TABLE 11

Showing the increase in the weight of the body with age for 99 male and for 76 female rats belonging in the seventh to the fifteenth generations of the A series of inbreds

AGE	MALES				FEMALES			
	Body weight in grams			Number of individuals	Body weight in grams			Number of individuals
	Average	Highest	Lowest		Average	Highest	Lowest	
days								
13	17.9	21	15	99	16.1	22	14	76
30	45.6	82	35	99	41.8	67	33	76
60	123.0	176	72	95	·102.3	159	71	74
90	189.8	248	120	99	150.3	193	93	63
120	233.9	305	174	94	170.0	212	133	63
151	263.4	360	204	94	187.6	224	157	65
182	287.4	367	216	91	197.5	236	167	62
212	296.8	420	238	85	202.8	248	158	59
243	309.5	419	234	84	209.9	245	171	56
273	322.6	435	265	74	217.0	251	167	53
304	321.1	415	267	70	221.0	257	181	42
334	328.9	415	268	63	227.2	268	196	37
365	336.2	428	289	59	229.8	282	198	35
395	335.9	433	283	51	230.3	274	206	31
425	345.9	448	281	44	232.2	268	206	21
455	361.3	473	306	25	228.3	257	182	20

TABLE 12

Showing the increase in the weight of the body with age for 57 male and for 93 female rats belonging in the seventh to the fifteenth generations of the B series of inbreds

AGE	MALES				FEMALES			
	Body weight in grams			Number of individuals	Body weight in grams			Number of individuals
	Average	Highest	Lowest		Average	Highest	Lowest	
days								
13	19.8	27	16	57	18.0	25	14	93
30	48.6	75	35	57	45.1	72	30	93
60	148.5	190	110	57	115.2	147	80	.93
90	210.1	266	149	57	161.1	197	117	68
120	254.1,	331	218	55	176.8	198	134	.73
151	282.9	365	232	55	193.4	229	156	67
182	307.5	407	253	52	213.7	241	168	70
212	319.4	410	254	44	208.2	247	174	60
243	323.1	408	259	39	212.5	261	179	54
273	330.7	454	275	32	213.3	278	177	47
304	344.3	477	290	28	221.1	281	179	40
334	344.4	385	299	18	230.3	290	179	26
365	348.2	376	298	17	233.7	276	194	29
395	355.1	374	333	11	237.2	301	206	23
425	353.2	365	339	5	239.4	323	210	19
455	336.6	347	320	3	° 245.0	317	212	13

Fig. 8 Graphs showing the increase in the weight of the body with age for males belonging in the seventh to the fifteeth generations of the two series of inbreds (data in table 11 and in table 12).
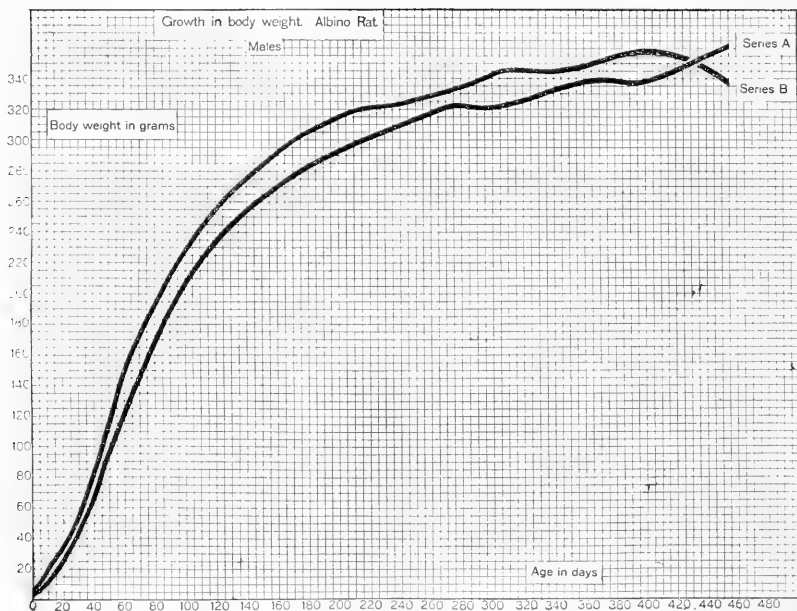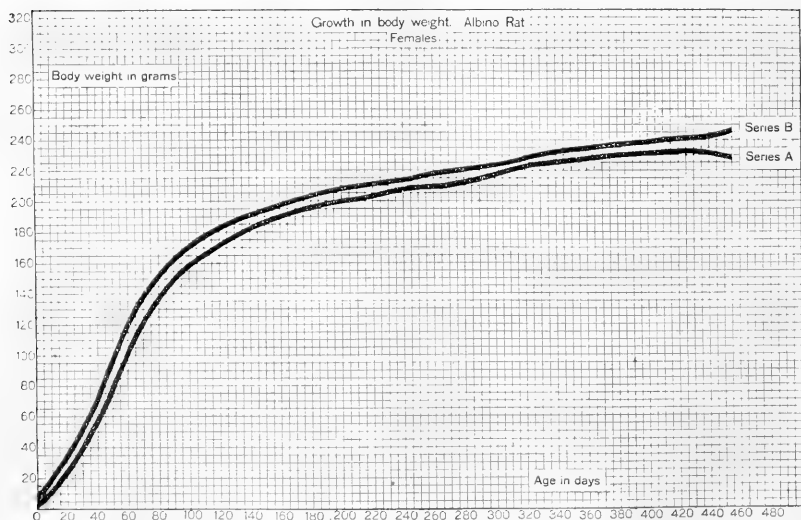

Fig. 9 Graphs showing the increase in the weight of the body with age for females belonging in the seventh to the fifteenth generations of the two series of inbreds (data in table 11 and in table 12).

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The graphs in figure 9 run close together throughout their entire course, but the graph for the B series is higher at all points than that for the A series. It appears, therefore, that growth in body weight was somewhat more vigorous in the females of the B series than in those of the A series.

It seems a rather significant fact that in both figure 8 and figure 9 the graphs run parallel from the beginning until the end of their course; they do not cross and recross at various points, as one might expect would be the case with graphs for two series of rats from the same ancestral stock that were reared simultaneously under the same environmental conditions. Female B and her mate, the ancestors of the B series of inbreds, were heavier animals at the time that they were killed than were the progenitors of the A series of inbreds, female A and her mate. Body weight in the rat is so dependent on physical condition, however, that a single weighing of the animals when they were at an advanced age would not necessarily give a true idea of the relative size of the animals at an earlier age period. The difference in the size of the two pairs of rats with which the experiments were started, together with the fact that after the sixth generation the descendants of female B were relatively heavier animals than the descendants of female A, point to the conclusion that the difference in the size of the animals in the two series was not due to chance or to environment, but that it was dependent in some way upon the inheritance of genetic factors for growth.

Table 13 gives the body weight data for the total of 156 males and 169 females in the seventh to the fifteenth inbred generations for which weight records were taken (a combination of the data in table 11 and in table 12).

Table 13 brings out one fact of interest: the average body weight of the male inbred rats increased with age up to the end of the weighing period when it was 358.7 grams; the average body weight of the females was at its maximum at the 425 day period, and then fell off slightly at the final weighing. Individual rats show a pronounced difference as regards the time that they attain their maximum body weight and, as a rule, the

Showing the increase in the weight of the body with age for 156 male and 169 female rats belonging in the seventh to the fifteenth generations of the two series (A, B). A combination of the data in table 11 and in table 12

	MALES				FEMALES			
AGE	Body weight in grams			Number of individuals	Body weight in grams			Number of individuals
	Average	Highest	Lowest		Average	Highest	Lowest	
days								
13	18.6	27	15	156	17.0	25	14	169
30	46.5	82	35	156	43.6	72	30	169
60	132.6	190	72	152	109.4	159	71	167
90	197.3	266	120	156	155.9	197	93	131
120	241.4	331	174	149	173.7	212	133	136
151	270.6	365	204	149	190.5	229	156	132
182	294.7	407	216	143	200.8	241	167	132
212	304.5	420	238	129	205.8	248	158	119
243	313.8	419	234	123	211.2	261	171	110
273	325.0	454	254	106	215.3	278	167	100
304	327.7	477	267	98	221.0	281	179	82
334	332.3	415	268	81	228.4	290	179	63
365	338.8	428	289	76	231.5	282	194	64
395	339.3	433	283	62	233.2	301	206	54
425	346.6	448	281	49	235.6	323	206	40
455	358.7	473	306	28	234.9	317	182	33

females reach this point at an earlier age than do the males. The records for these inbred rats show that in many cases the maximum body weight in both sexes came when the animals were seven or eight months of age, at which time they were at the height of their reproductive activity (King, '16), and then gradually decreased; other rats increased steadily in body weight until they were sixteen or even eighteen months old. Autopsies made on a considerable number of unusually large rats indicate that the later weight increase is chiefly an accumulation of adipose tissue and is not, therefore, to be considered as true growth.

Under the very favorable climatic conditions of California, Slonaker ('12 a) found that the albino rat reaches its maximum body weight, as a rule, by the age of fifteen months. In one

series of animals whose weight records were taken at frequent intervals from the time of weaning until natural death, Slonaker found that the ten males attained their average maximum body weight of 247.5 grams when they were 434 days of age, and that the six females reached their maximum weight of 151.8 grams somewhat earlier than did the males i.e. at 375 days of age. After the maximum was reached there was a slow but steady
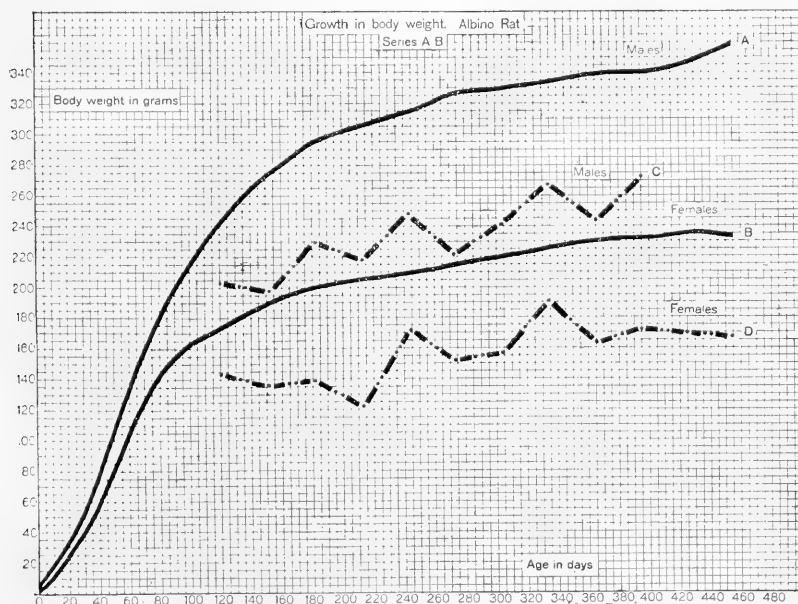

Fig. 10 .Graphs showing the increase in the weight of the body with age for males and for females belonging in various generations of the two series combined (A, B). Data in table 3 and in table 13. A and B, graphs for individuals in the seventh to the fifteenth generations inclusive; C and D, graphs for animals in the first six inbred generations.

decline in body weight, although the animals appeared to be in good physical condition, and some of them lived to be nearly four years old.

The graphs in figure 10 show the weight increase with age for the total number of males and females in the two inbred series for which weight records were obtained (data in table 3 and in table 13).

The graphs in figure 10 show in a very striking manner the great difference between the size of the rats in the first six inbred generations and those in the later generations. At the 300 day period the space between graph A and graph C represents a difference of 87 grams in the average body weights of the two groups of males. Females of the earlier generations (graph D) were likewise far inferior in body size to the females of subsequent generations (graph B), and at 300 days of age the space between graph B and graph D indicates a difference of 64 grams in favor of the females in the later generation group.

The earliest data on the growth in body weight of the albino rat are those of Donaldson ('06) who studied the growth changes in a series of animals reared at The University of Chicago. Other investigators, Jackson, Slonaker, Ferry and Hoskins have published records for the growth in body weight of various series of albino rats reared under different environmental conditions. All of the latter data agree, in the main, with those of Donaldson, although as the rat is very responsive to external conditions some series of records show more rapid and vigorous growth than others.

Donaldson's growth graphs for albino rats may be taken as representing the average run of stock animals. His graph for the males is reproduced as graph A in figure 11, and that for the females is shown as graph A in figure 12.

As controls for the present series of inbred albino rats thirteen litters of stock albinos, comprising fifty males and fifty females, were reared in The Wistar Institute animal colony under the same environmental and nutritive conditions as the later generations of the inbred series. In selecting litters for controls care was taken to pick out only those in which the young were large and vigorous at birth. The animals chosen, therefore, represent the best, not ,the average, stock in The Wistar colony. The average body weights at various ages of the males and females in this selected group of stock albinos, reproduced from table 3 of a previous publication (King, '15 a), are given in table 14.

Figures 11 and 12 show graphs for weight increase with age in two series of stock albinos (graphs A and C), together with graphs representing the growth of animals in the inbred series (graphs B and D). The graphs for the various groups of males are given in figure 11.
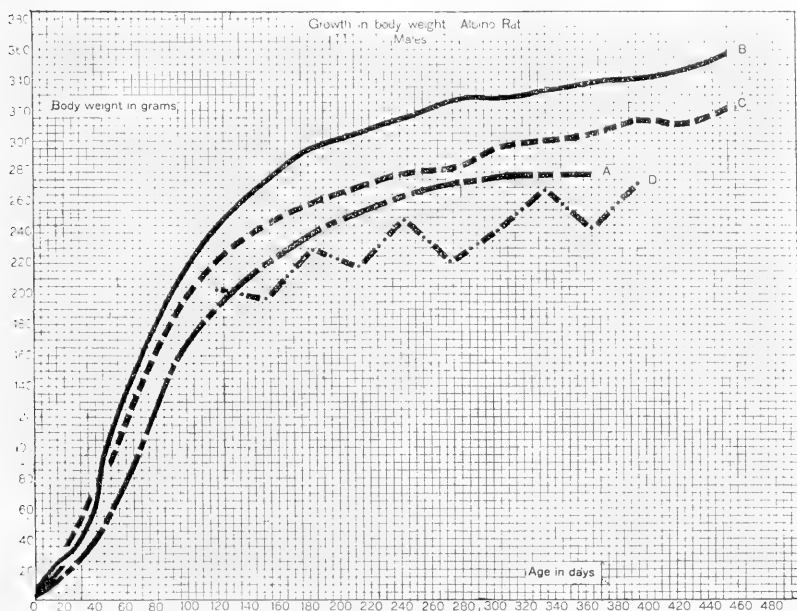

Fig. 11   Graphs showing the increase in the weight of the body with age for different series of male albino rats. A, graph constructed from Donaldson's data for stock albinos; B, graph for males belonging in the seventh to the fifteenth generations of the two series of inbreds combined; C, graph constructed from data for a selected series of stock albinos used as controls for the inbred strain; D, graph for males belonging in the first six generations of the two series combined.

In figure 11 graph B, representing the growth of the males in the inbred series, runs higher than Donaldson's graph for stock albinos (A) from the beginning until the end of its course. At the 243 day period the space between these graphs represents a difference of about 18 per cent in the average body weights of the two series of animals. At all points, except the thirty day period, graph B is higher than graph C which shows the body

growth of the selected stock males reared as controls for the
inbred group.   Data in table 13 and in table 14 show that at 13
days of age the inbred males weighed, on the average, 1.4 grams
more than did the stock males of the same age, but that at thirty
days of age the average body weight of the stock group was two
grams more than that of the inbreds; after this age inbred males
increased in body weight much more rapidly than did the stock
animals.   When the rats were at their prime, at eight months
of age, inbred males were about 12 per cent heavier than the
males of the control series.

The above analysis of data shows that not only were inbred
males of the seventh to the fifteenth generation much heavier
than the general run of stock animals at any given age, but that
they were also larger, except at the thirty day period, than
the selected stock controls reared under similar environmental
conditions.

Graphs showing the weight increase with age for various
groups of stock and inbred females are given in figure 12.

In figure 12 graph A, which indicates the body growth of the
females of Donaldson's series of stock albinos, is not strictly
comparable to the other graphs, since it was constructed from the
actual weight data for unmated females only up to the ninety
day period, beyond this point the data used were those of un-
mated females corrected to accord with the weights of breeding
females as calculated by Watson's ('05) formula: all other
graphs were based on the actual weight data of breeding females.
Graph A runs lower than either the graph for the inbred females
(B) or that for the stock controls (C) during the period in which
the actual weight records were used in constructing the graph,
but later it is considerably higher than the other graphs.   It
would seem as if the corrective factor introduced in Watson's
formula was much too high, since no series of actual weight
records for the albino rat yet reported comes up to the standard
required by Donaldson's graph.

In figure 12 the graph for the growth of inbred females (B)
and that for the females in the control series (C) run very close
together throughout their entire length.   From the thirty to

the sixty day period graph C is a little above graph B, but at all other points graph B is the higher of the two. At the 243 day period the space between the graphs represents a difference of only 0.76 per cent in the average body weights of the two groups of females, although the inbred females were 3.7 per cent heavier than the stock females when the animals reached one year of age.

These results indicate that the rate and the extent of the growth in inbred females was about the same as that of the females in the selected stock controls during the adolescent period,
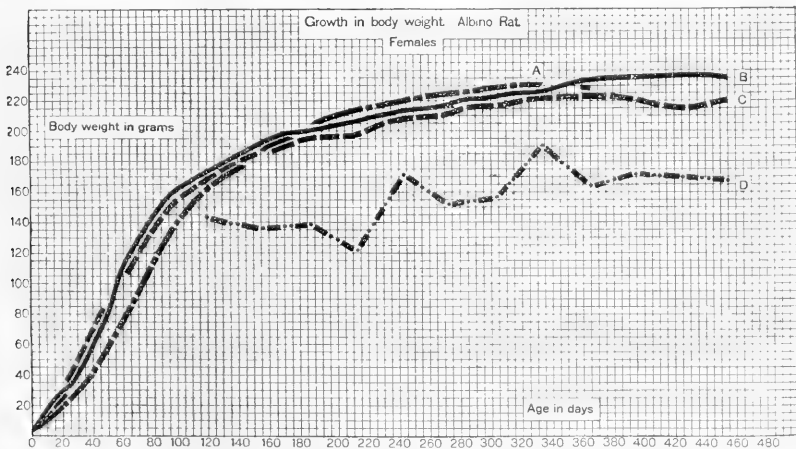
Fig. 12   Graphs showing the increase in the weight of the body with age ⸢for different series of female rats (lettering as in figure 11).

but that in the adult state the inbred females tended to be slightly heavier than stock females of the same age.

As shown in various tables (4–10) and in several figures (nos. 2, 4, 6) rats belonging to the later inbred generations were not as heavy at any given age as the animals in the seventh to the ninth inbred generations. One naturally asks whether inbreeding has lessened the growth impulse and impaired the vitality after many generations so that these animals are inferior in body size to normal stock animals of like age. In order to answer this question, data showing the increase in the body weight

TABLE 14

Showing the average increase in the body weight with age for two series of albino rats: (1) rats belonging to the fifteenth generation of the two inbred series (A, B); (2) selected stock rats. Both series of rats were reared under similar environmental conditions

	AVERAGE BODY WEIGHT IN GRAMS		AVERAGE BODY WEIGHT IN GRAMS	
AGE	Males	·	Females	
	Inbreds 21 rats	Stock 50 rats	Inbreds 27 rats	Stock 50 rats
days				
13	19.1	17.2	18.1	15.7 ·
30	44.9	48.5	42.8	45.7
60	132.3	122.9	106.2	107.1
90	184.9	184.8	147.9	148.0
120	216.1	223.2	168.2	173.4
151	249.1	244.8	184.1	186.3
182	269.0	258.4	202.4	196.5
212	278.3	268.0	208.0	197.3
243	291.3	279.7	210.6	209.6
273	302.0	280.9	218.0	210.8
304	305.3	296.1	219.8	219.1
334	309.8	300.8	225.4	222.4
365	318.8	306.1	227.3	223.1
395	315.8	314.1	226.2	220.5
425	323.2	312.2	227.4	215.8
455	319.7	323.9	226.3	220.2

with age in rats belonging to the fifteenth generation of the inbred series (A, B), with corresponding data for the animals in the Wistar stock control series are given in table 14.

Data for the two series of male rats, as given in table 14, are presented in the form of graphs in figure 13.

In figure 13 the graphs run close together and cross and recross until the 150 day period. At this point the graph for the inbred males mounts above that for the stock animals and subsequently maintains this position until the end, where the stock graph is the higher, since at this age the stock males were 1.3 per cent heavier, on the average, than the inbred males. At the eight months period the space between the graphs indicates a difference of 4.1 per cent in favor of the inbreds.

The majority of the inbred rats in the fifteenth generation were handicapped in their growth by being born in the summer:

Fig. 13 Graphs showing the increase in the weight of the body with age for males belonging in the fifteenth generation of the two series of inbreds combined (A, B), and for males in the series of stock controls.
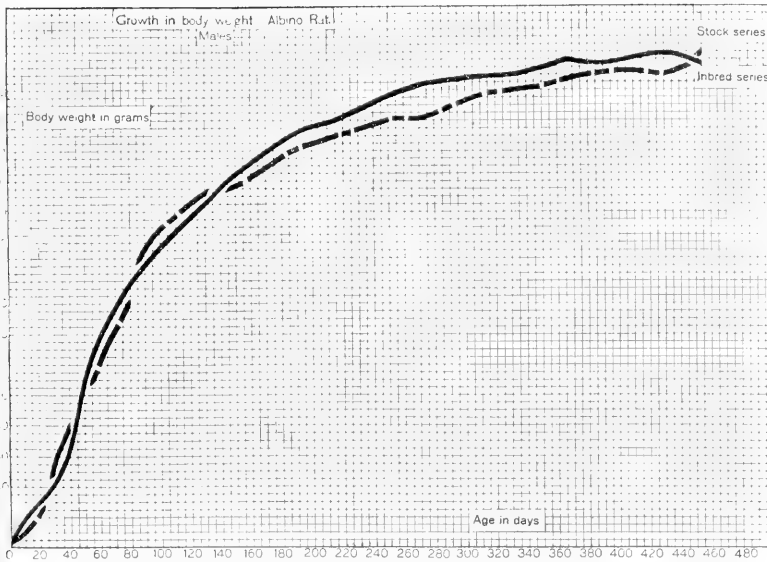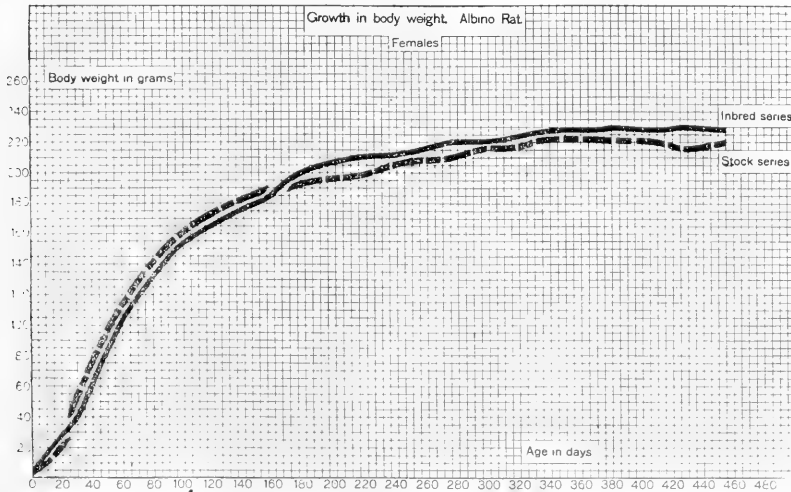

Fig. 14 Graphs showing the increase in the weight of the body with age for females belonging in the fifteenth generation of the two series of inbreds combined (A, B), and for females in the series of stock controls.

the stock rats had the advantage of birth in winter. Even under these conditions the males of the fifteenth inbred generation were, on the whole, heavier than the controls after they had attained maturity.

Figure 14 gives graphs showing the weight increase with age for females of the fifteenth inbred generation and for females of the stock control series.

A comparison of the growth graphs for the two groups of females (fig. 14) leads to the conclusion that there was no essential difference in the rate of growth of stock and of inbred females during the early growth period, but that in the adult state the inbred females were slightly heavier at any given age than the females in the control series.

3. VARIABILITY IN THE BODY WEIGHTS OF INBRED RATS

For the purpose of ascertaining the extent of variability in the body weights of the rats in the two inbred series, their coefficients of variation for the body weights at different ages were computed, together with the probable errors. Only records for animals belonging in the seventh to the fifteenth inbred generations were used for this purpose. No attempt was made to find the extent of variability in the body weights of the animals in the first six inbred generations, since only one weight for each animal was recorded and the age period covered by the data at hand was comparatively short.

Table 15 gives the coefficients of variation for the body weights at different ages, with their probable error, for the individuals in the seventh to the fifteenth generations of each of the two inbred series, and for the animals in the two series combined. Grouped data were used in making the calculations for the thirteen and for the thirty day periods, as only the average body weight of the individuals of each sex was recorded in the weighings of the various litters at these ages; for all other ages the individual data were employed.

Comparing the corresponding coefficients for the males and for the females it is found that in each series the female rats

TABLE 15

Showing the coefficients of variation, with their probable error, for the body weights at different ages of the two series of inbred rats (seventh to the fifteenth generation inclusive)

AGE	SERIES A		SERIES B		COMBINED SERIES (AB)	
	Males	Females	Males	Females	Males	Females
days						
13	8.0±0.39	11.8±0.64	11.7±0.74	11.2±0.56	13.2±0.51	12.4±0.45
30	16.6±0.80	16.2±0.88	14.8±0.93	17.4±0.86	16.2±0.62	18.3±0.67
60	19.9±0.96	20.1±1.17	12.8±0.88	14.2±0.70	18.2±0.69	17.7±0.65
90	13.8±0.66	15.6±0.93	11.6±0.73	11.6±0.67	13.8±0.53	13.9±0.58
120	12.9±0.63	10.7±0.64	11.9±0.76	7.6±0.42	12.9±0.51	9.2±0.37
151	11.4±0.56	8.8±0.52	9.2±0.60	7.7±0.45	12.0±0.48	8.9±0.37
182	12.1±0.60	8.0±0.48	11.0±0.72	8.3±0.47	10.2±0.41	8.2±0.34
212	11.4±0.59	9.2±0.57	11.1±0.79	7.7±0.47	12.2±0.51	8.5±0.37
243	11.9±0.62	8.2±0.52	11.4±0.86	8.5±0.55	11.6±0.50	8.1±0.37
273	12.6±0.69	9.0±0.59	11.6±0.97	10.0±0.69	12.3±0.57	9.3±0.45
304	10.7±0.61	8.6±0.64	12.7±1.14	9.5±0.72	11.9±0.57	9.2±0.48
334	9.9±0.60	8.2±0.68	7.9±0.88	9.1±0.85	10.9±0.58	8.5±0.51
365	11.0±0.68	9.0±0.72	6.4±0.74	8.1±0.72	10.5±0.58	8.6±0.51
395	10.6±0.71	7.7±0.66	4.3±0.62	9.2±0.91	9.9±0.60	8.6±0.56
425	11.9±0.85	7.9±0.82	3.0±0.64	10.9±1.19	9.8±0.67	10.9±0.87
455	14.3±1.36	9.6±1.03	3.4±0.98	11.5±1.52	13.7±1.23	11.0±0.91
Average......	12.4±0.70	10.5±0.71	9.7±0.81	10.1±0.73	12.4±0.56	10.7±0.52

were somewhat more variable in body weight than the males during the early stages of development up to sixty days of age, and that beyond this point the males were the more variable. In each series, also, the maximum variability in body weight came at the same age for both sexes: but this maximum was at the sixty day period in the A series of inbreds and at the thirty day period in the B series. The coefficients indicate, moreover, that there was a pronounced tendency in each sex for the variability to diminish after the period of rapid growth was ended. Guinea-pigs show a similar decrease in variability in body weight with advancing age, as was noted by Minot ('91).

The average coefficient for the male groups of the A series, taking all ages together, was about two points higher than the corresponding coefficient for the females of this series. This dif-

ference is nearly three times the probable error, and is, therefore, large enough to signify that in this series the range of variability in the body weight of the males was greater than that of the females.

In the B series the cofficients for the males were, as a rule, larger than those for the females up to the 334 day period. Beyond this age the coefficients for the females were the larger. This latter relation is not a normal one, and it can be attributed in this instance to the fact that the number of records that were available for use in calculating the coefficients for the older males was very small: in this series only seventeen out of a total of fifty-seven males lived to be one year old.

At all age periods, except at 13 and at 304 days, the coefficients for the males of the A series of inbreds were higher than the corresponding ones for the males of the B series, although the difference in some cases was less than the probable error. Between the average coefficients for the two male groups there was a difference of 2.7 points, but the importance of this difference is greatly lessened by the fact that the coefficients for the older males in the B series were abnormally low. The evidence, on the whole, would seem to indicate that there was little difference in the range of variability in the body weights of the males in the two inbred series, since a comparison of the average coefficients for the two male groups up to 334 days, taking all ages together, shows a difference of only 1.1 points in favor of the individuals in the A series.

At ten of the sixteen age periods shown in table 15 the coefficients of variation for the females of the B series of inbreds exceeded those for the females of the A series, but in many cases the differences were less than the probable error and therefore they can have no significance. The average coefficients for the two groups differed by only 0.04 points, so it is evident that the range of variability in body weights was practically the same for the females of the two inbred series.

A comparison of the coefficients for the males with those for the females in the combined series (A, B) shows that in the majority of cases the male coefficients were much the higher.

At the thirty day period only was the coefficient for the female group significantly greater than the corresponding one for the male group. The greater variability in the body weights of the females at this age is doubtless correlated with the fact that during early postnatal life female rats are growing more rapidly than the males. From the evidence at hand it appears that inbred males are more variable in body weight than inbred females. Jackson ('13) and King ('15 a) have already noted that in groups of stock albinos the males tend to be more variable in body weight than the females at corresponding age periods.

As growth records were taken for only a comparatively small number of animals in each generation of the two inbred series, it did not seem advisable to calculate the coefficients of variation for each generation separately, since Pearson has shown that with numbers less than twenty-five the empirical standard deviation is usually too small. The combined records for the individuals of the two series (A, B), divided into three groups as shown in table 10, were used in calculating the coefficients of variation for the body weights of the generation groups as given in table 16.

As table 16 shows, corresponding coefficients for the three generation groups varied considerably in some cases, but there was a decided tendency in both sexes for all the coefficients to become smaller as the inbred generation advanced. The difference between the average coefficients for the males and for the females in successive generation groups was about two points in each instance. It appears, therefore, that variability in body weight diminished at a fairly uniform rate from one inbred generation to the next. The average decrease for each generation was comparatively slight, amounting to less than one per cent, and it was about the same for the two sexes, although in all generation groups the males tended to be somewhat more variable in body weight than the females.

The question arises as to how the variability in the body weight of the inbred rats compares with that in stock animals in which there is no inbreeding. Obviously in this instance one should compare inbred and stock animals taken from the same strain and reared under similar environmental conditions, since,

TABLE 16

Showing the coefficients of variation, with their probable error, for the body weights at different ages of inbred rats of the two series combined (A, B) separated into three groups according to the generation to which the individuals belonged

AGE	MALES			FEMALES		
	Generations 7–9	Generations 10–12	Generations 13–15	Generations 7–9	Generations 10–12	Generations 13–15
days						
13	13.0±1.06	11.2±0.70	9.4±0.46	14.9±1.09	10.6±0.65	14.4±0.84
30	22.7±1.85	18.4±0.53	11.8±0.69	21.7±1.58	14.8±0.92	15.5±0.91
60	22.9±1.88	21.1±1.46	14.8±0.88	18.8±1.36	16.4±1.32	14.0±0.82
90	15.5±1.26	13.2±0.76	12.4±0.74	18.6±1.52	13.0±0.98	11.4±0.72
120	15.5±1.32	11.8±0.76	11.7±0.70	11.4±0.99	7.5±0.53	9.2±0.56
151	15.1±1.25	11.8±0.76	9.4±0.57	12.2±1.17	7.3±0.50	8.5±0.52
182	13.6±1.13	10.8±0.74	8.6±0.52	10.1±0.87	8.0±0.56	7.1±0.45
212	12.9±1.15	11.1±0.82	8.8±0.55	9.2±0.84	10.3±0.88	6.8±0.41
243	10.8±0.97	11.9±0.88	8.2±0.53	9.5±0.92	9.1±0.80	7.3±0.46
273	12.3±1.17	10.8±0.89	8.7±0.59	11.4±1.21	10.5±1.00	7.6±0.49
304	10.7±1.13	11.3±0.96	9.9±0.69	10.0±1.32	12.1±1.29	7.3±0.49
334	7.8±0.80	9.7±1.03	9.3±0.69	8.6±1.23	11.2±1.38	6.9±0.54
365	6.1±0.68	9.5±0.94	8.6±0.69	9.7±1.64	10.8±1.25	6.5±0.49
395	9.8±1.20	8.5±0.96	6.4±0.56	10.6±2.06	11.3±1.50	5.5±0.44
425	10.6±1.51	8.2±1.01	7.4±0.73	12.3±3.40	10.3±1.64	5.9±0.51
455	10.5±1.57	5.0±0.61	6.0±0.94		7.5±1.26	7.9±0.79
Average......	13.1±1.24	11.5±0.86	9.5±0.66	12.6±1.41	10.7±1.02	8.9±0.59

as several investigators have shown, albino rats from diverse strains that are reared in various ways show pronounced differences in their rate of growth, and presumably there is a corresponding difference in the variability of their body weights at different ages.

Table 17 gives the coefficients of variation for body weights at various ages of fifty male and of fifty female rats reared as controls for the present inbred series. As already stated these animals were a selected group taken from the same strain as the inbred rats and reared under similar environmental conditions. The coefficients of variation for stock rats given in table 17 are reproduced from tables 4, 5 and 6 of a previous publication (King, '15 a).

TABLE 17

Showing the coefficients of variation with their probable error for the body weights at different ages of male and female rats belonging to three series: (1) eight litters of the fifteenth generation of inbreds (series A, B); (2) thirteen litters of selected stock; (3) a single litter of selected stock

	MALES			FEMALES		
AGE	(1) Inbreds 21 rats from 8 litters (15th genera- tion)	(2) Stock 50 rats from 13 litters	(3) Stock 9 rats from 1 litter	(1) Inbreds 27 rats from 8 litters (15th genera- tion)	(2) Stock 50 rats from 13 litters	(3) Stock 7 rats from 1 litter
days						
13	10.4 ± 1.08	11.8 ± 0.79		9.4 ± 0.86	11.4 ± 0.76	
30	10.5 ± 1.08	10.2 ± 0.68		9.3 ± 0.86	11.0 ± 0.74	
60	14.2 ± 1.47	17.0 ± 1.14	9.6 ± 1.52	11.9 ± 1.09	15.7 ± 1.05	6.4 ± 1.15
90	12.9 ± 1.33	14.8 ± 0.99	7.9 ± 1.25	13.2 ± 1.37	12.5 ± 0.95	9.2 ± 1.78
120	11.8 ± 1.23	13.4 ± 0.90	6.5 ± 1.03	8.8 ± 0.86	10.3 ± 0.75	6.5 ± 1.16
151	9.8 ± 1.01	13.3 ± 0.89	5.1 ± 0.80	6.2 ± 0.58	10.4 ± 0.73	8.9 ± 1.59
182	9.1 ± 0.94	14.2 ± 1.22	7.1 ± 1.12	6.7 ± 0.67	12.3 ± 0.90	6.6 ± 1.40
212	8.9 ± 1.00	14.0 ± 0.96	6.8 ± 1.14	5.1 ± 0.48	12.4 ± 0.91	4.9 ± 1.04
243	8.2 ± 0.98	13.9 ± 0.99	10.1 ± 1.69	6.4 ± 0.64	12.6 ± 0.91	7.7 ± 1.38
273	7.5 ± 0.93	13.4 ± 0.99	9.5 ± 1.59	5.4 ± 0.58	11.5 ± 0.89	8.5 ± 1.53
304	5.8 ± 0.72	14.0 ± 1.11	10.0 ± 1.68	3.6 ± 0.40	10.3 ± 0.79	7.4 ± 1.32
334	6.1 ± 0.78	13.7 ± 1.13	8.6 ± 1.44	3.5 ± 0.42	10.8 ± 0.87	6.3 ± 1.22
365	4.0 ± 0.55	13.0 ± 1.16	10.0 ± 1.68	5.2 ± 0.61	10.7 ± 0.91	5.3 ± 1.22
395	4.5 ± 0.67	12.6 ± 1.22	9.8 ± 1.65	4.4 ± 0.57	11.5 ± 0.98	6.5 ± 1.68
425	4.9 ± 0.83	13.4 ± 1.32	13.3 ± 2.23	5.2 ± 0.69	10.9 ± 0.94	5.4 ± 1.15
455	1.6 ± 0.39	13.6 ± 1.67	13.7 ± 2.66	7.5 ± 1.13	8.9 ± 0.99	6.0 ± 1.16
Average......	8.1 ± 0.99	13.5 ± 1.07	9.1 ± 1.53	6.9 ± 0.73	11.4 ± 0.88	6.8 ± 1.34
Average omitting first two records....	7.8			6.6		

A comparison of the coefficients for the males of the combined series (table 15) with the corresponding coefficients for stock males (table 17) shows that inbred males tended to be somewhat more variable in body weight than stock males up to sixty days of age, which is the period of maximum variability for both groups. From this age on, however, stock males were apparently more variable than inbred males. The average coefficient

for stock males, taking all ages together, was 13.5, while that for
the inbred males was 12.4. Since the difference between these
coefficients is only slightly greater than the probable error, it is
evident that close inbreeding did not decrease the variability
of the entire male population more than about 8 per cent.

When the coefficients for the inbred females of the combined
series (table 15) are compared with those for stock females
(table 17) it is found that in this sex, also, inbred animals were
more variable in body weight than stock animals during the early
growth stages. After the age of ninety days, however, stock
females tended to be slightly more variable in body weight than
inbred females. The average coefficients for the two groups
differ by less than one point, so it appears that the inbred females
had practically the same range of variability in body weight as
the stock females.

In a study of the variability in body weight of the albino
rat, Jackson ('13) found that: "variability in body weight is
lowest at birth (the coefficient being about 12) and is not much
higher at seven days (16). It appears highest at three weeks
(28), and at later periods varies from 19 to 21. The average co-
efficient, taking all ages together, is 19." In Jackson's series the
maximum variability in body weight comes at an earlier period
than it does in either the inbred series or in the stock controls,
and his coefficients are much higher for all ages. The fact that
Jackson used data obtained from rats that represented "for the
most part a random sample of the general population at each
age" undoubtedly accounts for the rather wide range in our re-
sults, although a difference in the strain of albinos used and in
the environmental conditions to which the rats were subjected
may have contributed to the result.

In order to determine whether the animals in the later inbred
generations were any less variable in body weight than those in
the general inbred population, the coefficients of variation for the
body weights at different ages of individuals in eight litters of
the fifteenth inbred generation were determined and are given
in table 17. All of these coefficients were decidedly lower than
the corresponding ones for the individuals of the combined (A, B)

series of inbreds (table 15). The difference between the average coefficients was quite large, amounting to 4.3 points for the males and to 3.8 points for the female groups. This result indicates that the animals of the fifteenth inbred generation were about 35 per cent less variable in body weight than the animals in the general inbred population.

When the coefficients of variation for the individuals in the fifteenth inbred generation are compared with those for the stock controls (table 17) the results are equally striking and significant, At only one age period, i.e., thirty days, was the coefficient of variation for the inbred males slightly in excess of that for the stock males; at all other ages the coefficients for the stock males were much the larger. The average coefficient for the stock males was 13.5, while that for the inbred males was only 8.1. It appears, therefore, that the variability in the body weights of the males of the fifteenth inbred generation was, on the average, about 40 per cent less than that in stock males.

Judging from the size of the corresponding coefficients (table 17) females belonging to the fifteenth inbred generation were more variable in body weight at ninety days of age than the females of the stock controls, at all other ages the stock females were the more variable. The difference of 4.5 between the average coefficients for the two groups indicates that the females of the fifteenth inbred generations were about 40 per cent less variable in body weight than stock females.

From a study of fraternal variability in the albino rat, Jackson concludes that "in general the variation in body weight within a given litter of albino rats is probable less than half that of the general population of the same age under similar environment." In the series of stock animals reared as controls for the present inbred series it was shown (King, '15 a) that "the range of variability within the litter is about 70 per cent that of the general population in the case of the males, while for the females it is about 55 per cent."

By comparing corresponding coefficients, as given in table 17, it is possible to determine whether variability in the body weight of the animals in the fifteenth inbred generation was greater or

less than fraternal variability in stock animals.   The evidence as given indicates that the inbred males were more variable in body weight than the males in a single stock litter up to 212 days of age, but that at all subsequent ages stock males were very much more variable.   Omitting from the inbred series the co-efficients for the first two age periods, since there were no corre-sponding coefficients for the males of the stock litter, the average coefficient for the males in the fifteenth inbred generation, tak-ing all ages together, was 7.8, while the average coefficient for the stock males was 9.1.   The difference between the coefficients was not very great, but it seems large enough to signify that the variability in the body weights of the males in the fifteenth in-bred generation was somewhat less than fraternal variability in the stock controls.

The relative variability of inbred and stock females was slightly different from that found in the male groups.   Females of the fifteenth inbred generation were, as a rule, more variable in body weight than females in a single stock litter up to 120 days of age, beyond this age stock females seemed to be the more variable.   The difference of 0.2 points between the aver-age coefficients for the two groups, omitting the findings for the first two weighings of the inbred group, was in favor of the stock litter.   This difference, however, is much too small to have any meaning, and it is evident that the variability in the body weights of the females of the fifteenth inbred generation was about the same as fraternal variability in the stock controls.

4. DISCUSSION OF RESULTS

This study of the growth in body weight of albino rats belong-ing to fifteen generations produced by brother and sister mat-ings has shown that the closest form of inbreeding possible in mammals does not necessarily produce animals that are below the normal body size, as Crampe ('83) and Ritzema-Bos ('93; '94) have maintained.   During the early part of these experiments all of the evil effects that are said to follow from close inbreed-ing were obtained, but it was shown conclusively that they

were not due to inbreeding but solely to malnutrition. There is a possibility, therefore, that many of the bad results obtained in other inbreeding experiments with rodents may have been produced, in part at least, by unfavorable environmental or nutritive conditions. The published records of the former work give no details regarding the manner in which the experiments were conducted, consequently there is no way of determining to what extent external conditions were responsible for the outcome. Both Crampè and Ritzema-Bos worked with hybrids, which frequently exhibit a tendency to sterility as others have noted, and the animals were inbred promiscuously for the most part. Even the most favorable environmental conditions could not be expected to keep such animals up to normal standards for body size or for fertility.

In the present series of experiments improper feeding through four successive generations did not permanently impair the growth power of the individuals, which responded at once to the stimulus of a well balanced diet. The rats in the fifth and those in the sixth inbred generations grew much more vigorously than their forefathers, and many of them attained an adult size equal to that which is normal for the albino rat.

The maximum effect of the stimulus given to the growth impulse by adequate nourishment did not seem to be reached until the seventh generation when some of the animals were larger than any albino rats as yet recorded. In the two following generations the average body weight of both males and females at various age periods decreased slightly, but they were still far above the norms for stock animals and higher than the averages for the individuals in the eleventh and succeeding generations. From the tenth to the fifteenth generations there was no very marked change in the average body weights of the rats at corresponding age periods, but the body weights tended to decrease slightly as the inbreeding advanced.

It seems probable that the exceptionally vigorous growth of the rats in the seventh to the ninth inbred generations was wholly, or in great part, a response of the organisms to very favorable nutritive conditions following a period of partial starvation.

Hatai ('07), Osborne and Mendel ('14; '15; '16) and Stewart ('16) have shown that the growth of the albino rat can be inhibited for varying periods, either by starvation or by improper food, and that there is at once a resumption of growth when a return is made to a normal diet; the animals eventually reaching the size of the controls or even surpassing them in body weight at corresponding ages.

Although, as a rule, adult rats increase in body weight very slowly and may even remain at practically the same body weight for several successive months, this slowing up of the growth process is apparently not due to an exhaustion of the growth capacity. The extensive experiments of Osborne and Mendel show that the capacity to grow can be retained and exhibited at periods far beyond the age at which growth ordinarily ceases, and their work points to the conclusion that in the rat "the capacity to grow is only lost by the exercise of this fundamental property of animal organisms." What is true for the individual may also be true for the race. The capacity to grow is seemingly so essential a part of the organism that this power is retained through several successive generations in which it is not exercised to its full extent. In this series of experiments, as far as is known, not a single individual of the many hundreds that were reared in the first four generations attained a body size that equaled the norm for the adult albino rat. Yet even after this long period of time the growth impulse in all individuals at once responded to the stimulus of adequate nutrition, and only two generations were required to effect a return to normal body size.

That favorable nutritive conditions had produced a parallel modification of the soma and of the germplasm might be a satisfactory explanation for the appearance of the exceptionally large individuals in the seventh to the ninth inbred generations were it not for the fact that this increase in the body size of the individuals was temporary, lasting through these generations only. It seems more probable that favorable nutritive conditions, following a period of semi-starvation, greatly increased metabolic activity and so stimulated the growth impulse that the

animals attained an unusually large size. After the maximum effect of the stimulus had passed there was a gradual decline to more normal conditions of metabolism and a corresponding decrease in the average size of the individuals. I see no reason to assume that the hereditary factors concerned in growth were influenced either by malnutrition during the early part of the experiment, or by favorable nutrition in the later generations.

Crampe ('83) and Hoskins ('16) have noted that the growth of albino rats is influenced to a considerable extent by the time of year in which the animals are born. Rats born in the winter months are larger at a given age, live longer and are more vigorous than those born in the summer or autumn. The superiority of the winter-born rats has been most marked in the various breeding experiments that I have been carrying on for several years with different strains of rats. It is not improbable that the rats of the seventh inbred generation owed some part of their vigorous growth to the fact that they were born in the most favorable season of the year, the early winter months. The environmental agency here concerned in stimulating growth is either temperature or humidity, possibly both. A moderate degree of cold is apparently more conducive to rapid and vigorous growth in rats than is heat: the reverse is true for the mouse, according to the investigations of Sumner ('09; '15). Extreme temperature, either heat or cold, has a very unfavorable effect on the rat, making the animals exceeding susceptible to the rat scourge, pneumonia, which invariably proves fatal to an animal of any age.

In these experiments, as already stated, there was a very careful selection of breeding stock from the seventh generation on. Small, weak, inferior animals were eliminated before reaching maturity, and only the largest and most vigorous animals were allowed to perpetuate their kind. By this rigid selection it was possible to keep the animals up to a high standard for body size and to make the strain apparently immune to the injurious effects that would probably have followed from random matings. In other inbreeding experiments where selection of breeding animals was made on the basis of size and vigor there was no

marked deterioration in the stock. Castle ('16 a) inbred rats
within narrow lines of selection for seventeen successive gen-
erations and was able to maintain races 'with fair vigor and
fecundity.' Inbreeding experiments with Drosophila, carried on
for many generations by Castle et al. ('06) and by Moenkhaus
('11), have shown that in this form, also, races of large size and
vigor can be maintained under the closest inbreeding by simply
selecting the most vigorous parents for breeding.

Undoubtedly various species of plants and animals react dif-
ferently under inbreeding. In tobacco, inbreeding is "beneficial
and offers an effective means of maintaining desirable character-
istics in the established varieties" (Shamel, '05); while in maize
inbreeding leads to a considerable loss in vegetative vigor but
not to degeneration (East and Hayes, '11; '12). In swine, ac-
cording to Darwin ('76), close inbreeding invariably leads to
sterility and to a considerable loss in body size after only a few
generations, although this has recently been denied by Gentry
('05). From available evidence inbreeding seems to be very inju-
rious to dogs (Darwin, '76; B, '06), to pigeons (Fabre-Domengue,
'98) and to goats (Ewart, '10). On the other hand, it is chiefly
through inbreeding, with selection, that the thoroughbred types
of cattle, of sheep and of horses have been developed and fixed,
and there is no evidence of degeneration in the descendants
of deer and of rabbits that have been inbred in isolated com-
munities for many years (Ewart, '10). If pure stock that ful-
fills standard requirements as to size, vigor and fertility is used
for the investigation, and only vigorous, sound animals are
allowed to breed, there is, theoretically, no ground for believing
that continued inbreeding will cause either loss of vigor or a
decrease in body size. In these experiments with the rat, the
bad effects of inbreeding per se, as far as they might manifest
themselves by a decrease in the body size of the individuals, have
apparently been entirely prevented through the use of a strain of
animals that seemingly had no inherent defects and by a careful
selection of breeding stock. Even after twenty-eight genera-
tions of continued brother and sister matings the inbred animals
have not deteriorated in any way, and they are still superior

in body size to stock animals reared under similar environmental conditions.

Brother and sister matings automatically tend to reduce heterozygosis, and by the time that the animals have reached the fifteenth generation they are 96.277 per cent homozygous (Fish, '14). Such inbred individuals, according to Pearl ('13), can by no chance possess more than 0.006 of 1 per cent of the different lines of ancestral descent which are theoretically possible. In the present experiments selection was also a factor that tended to decrease heterozygosis, since the mating of only the largest and most vigorous pair of individuals in a litter presumably brought together gametes of like genetic constitution, in the majority of cases, and thus aided in increasing the proportion of homozygotes in the progeny population. In spite of the very high degree of homozygosis which they had attained, the animals of the fifteenth inbred generation showed a considerable amount of variability in body weight at different ages (table 17). How much of this variability was due to genetic factors for body size and how much was purely the result of environmental and nutritive action is not known, since there is, as yet, no means of determining to what extent environmental agencies can influence body growth. Nutritive conditions alone can greatly alter body size in the rat, as is shown by the experiments of Osborne and Mendel ('16). Temperature and humidity likewise act upon body growth in rodents (Sumner, '09; '15), and housing conditions are known to materially change their body size. With all of these environmental agencies that are known to affect body size made as uniform as possible under existing laboratory conditions, the variability in the body weights of the inbred rats continued to decrease at a small, but fairly uniform, rate in both males and females from the seventh to the fifteenth generation. This indicates that the individuals were becoming more homozygous with respect to the factors that determine body size. It is evident, however, that even after fifteen generations of continued brother and sister matings the strain was far from 'pure' in the sense in which this term is used by Johannsen ('09) and his followers, since the data for body growth in the

individuals of later generations, which will be given in a subsequent publication, show a still further decrease in the variability of body weights as the inbreeding advanced.

As the coefficients of variability were not determined for any individuals in the first six inbred generations, the extent of variability in the body weights of the inbred series as a whole is not known. A comparison of the average coefficients for body weight as given in table 15 and in table 17 show that the animals in the seventh to the fifteenth inbred generations were, as a group, about 7 per cent less variable in body weight than the animals in the control series. This difference would doubtless be greater if the inbred rats had not undergone unusual changes in body weight due to altered conditions of nutrition.

On the basis of the calculations made by Fish ('14) regarding the amount of homozygosis in different generations of animals produced by brother and sister matings, the group of rats comprising the seventh to the ninth inbred generation was, on the average, about 83 per cent homozygous. These animals, as table 16 shows, had a range of variability in body weight that was greater than that in any other generation group. It is interesting to note that, although the body weights of this group of inbreds greatly exceeded the norms for stock animals of like age, their variability was not correspondingly increased, since the average coefficients for the group are about the same as those for the stock controls (table 17). The group comprising the individuals of the tenth to the twelfth inbred generations was, according to Fish's table, about 91 per cent homozygous, while the last group was, on the average, 95 per cent homozygous. Since body weight probably depends to a considerable extent on "the presence or absence of definite genetic factors segregating from one another in gametogenesis on lines with which we are already familiar" (Punnet and Bailey, '14), one might expect to find a definite correlation between the amount of homozygosis and the variability in body weight in animals obtained from brother and sister matings. On referring to the average coefficients for the body weights of the various generation groups, as given in table 16, it is found that for both males and females

there was, in every case, a difference of about two points between the coefficients for successive generation groups. In these inbred rats, therefore, variability in body weight did not decrease proportionately as the homozygosis of the individuals increased. The results are complicated by the fact that part, at least, of the variability in body weight that was measured by the coefficients of variability was due to environmental action and cannot be distinguished from the variability due to genetic growth factors. When the data for a number of later inbred generations have been examined it may then be possible to ascertain the probable extent of variability due to environment and to correlate the amount of homozygosis in the individuals with the extent of their variability in body weight.

The records, as given above, show unquestionably that the variability in body weights of the individuals decreased as the inbreeding advanced. The results, therefore, do not accord with Walton's ('15) theory that continued inbreeding tends to increase rather than to diminish variability. In an able criticism of this theory Castle ('16 c) says: "It is difficult to understand how on any theory of heredity inbreeding could be expected to increase variability within a single inbred line. . . . . On a Mendelian theory it would be expected that inbreeding, brother with sister, for a large number of generations would result in the production of a number of homyzygous lines, each of which by itself would be entirely devoid of variability, except that due to environmental agencies." The results so far obtained in this inbreeding experiment with the rat are in harmony with Castle's view, though in such a complex organism as the rat it is not probable that any degree of inbreeding will produce lines that are "entirely devoid of variability, except that due to environmental agencies."

In discussing the effects of close inbreeding on Drosophila, Castle et al. ('06) state: "These experiments show no appreciable effect of inbreeding. In every case the brood reared under the best and most uniform conditions has the highest average number of teeth (on the sex comb), irrespective of whether or not inbred. The same may be said of variation in size. Inbreeding

has diminished neither the average size nor the variability in size.'' The reactions of the rat to close inbreeding are slightly different from those of Drosophila. The closest form of inbreeding possible, continued for many generations, has not caused a diminution in the average body weight of inbred rats at any age. On the contrary, through the selection of only the largest and most vigorous animals for breeding, inbred animals, especially the males, are superior in body size to the best stock animals reared under similar environmental conditions. In the rat variability in body weight decreases after inbreeding, and in the fifteenth inbred generation the variability was no greater than fraternal variability in the body weights of stock albinos.

The more general bearing of the results of these inbreeding experiments will be discussed in a following paper dealing with the fertility and constitutional vigor of inbred rats.

SUMMARY

1. The present paper gives an analysis of the data for the increase in the weight of the body with age for 333 male and for 306 female albino rats. These animals belonged to two series (A and B), both descended from the same ancestral stock, that were inbred, brother and sister only, for fifteen successive generations.

2. The animals of the first six generations suffered from malnutrition, and their body weights were much smaller than the norms for stock animals of like age. Many of these animals had defective teeth and the majority of the females were sterile. When nutritive conditions were improved the animals quickly regained their normal body size and the tendency to sterility and to malformation was checked.

3. The general course of the growth in body weight of inbred rats is similar to that of stock animals as determined by the investigations of Donaldson, Jackson and others.

4. In both inbred series the average body weights of the males was greater than that of the females at every age at which weighings were taken. The males and females of the B series

were somewhat heavier at all ages than the animals of the A series.

5. The males belonging in the seventh to the ninth generations of the two inbred series greatly exceeded the males of the first six inbred generations in body size, and they were, as a rule, much heavier, at all ages, than the males of the subsequent generations (fig. 6).

6. The females belonging in the first six generations of the two inbred series were considerably smaller, at all ages, than the females of the subsequent generations. Adult females of the seventh to the ninth inbred generations were slightly larger than the females of the later generations (fig. 7).

7. The unusual size of the individuals in the seventh to the ninth inbred generations was probably due, in great part, to the stimulus given to the growth impulse by favorable nutritive conditions following a prolonged period of semi-starvation.

8. Inbred males belonging in the seventh to the fifteenth generation inclusive were heavier at all ages than stock albinos. In the adult state the inbred males were, on the average, 18 per cent heavier than the general run of stock albinos and about 12 per cent heavier than males from a selected stock series reared under the same environmental condition (fig. 11).

9. Inbred females were, as a rule, slightly heavier at any given age than the females of the control series, but the difference between the two groups was much less than in the case of the males. At the 365 day period the average body weight of the inbred females was 3.7 per cent greater than that of the stock females (fig. 12).

10. Inbred males were more variable in body weight than inbred females, the maximum variability for both sexes coming before the animals were two months old. These results agree with the findings for stock albinos as determined by the investigations of Jackson and of King.

11. The males of the A series of inbreds were slightly more variable in body weight than the males of the B series, but the females of the two series showed practically the same variability in body weight at corresponding age periods (table 15).

12. In both series the variability in the body weights of males and of females decreased as the inbred generation advanced. The average decrease was about 2 per cent for a group of three generations (table 16).

13. Inbred males were more variable than stock males up to sixty days of age. After this time stock males showed greater variability in body weight at all ages (tables 15, 17).

14. Inbred females were more variable in body weight than stock females up to ninety days of age. After reaching maturity stock females tended to be slightly more variable in body weight than inbred females (tables 15, 17).

15. Males and females of the fifteenth inbred generation were about 35 per cent less variable in body weight than the animals of the general inbred population, and about 40 per cent less variable than the animals in the series of stock controls (table 17).

16. The variability in the body weights of the males of the fifteenth inbred generation was somewhat less than fraternal variability in the males of the stock controls. In the corresponding female groups variability in body weight was practically the same at all ages (table 17).

RUBY-EYED DILUTE GRAY, A THIRD ALLELOMORPH IN THE ALBINO SERIES OF THE RAT

P. W. WHITING AND HELEN DEAN KING

*From the Zoological Laboratory of the University of Pennsylvania and The Wistar
Institute of Anatomy and Biology*

INTRODUCTION

The discovery of a ruby-eyed dilute variety of the Norway rat
was announced by Whiting in 1916. As was stated at the meet-
ings of the American Society of Naturalists in December, 1916,
the factor responsible for this variation has proved to be a third
allelomorph in the albino series.

The dilute rats were taken near the University of Pennsylvania
in the spring of 1916. It may be well to give a detailed account
of the occurrence of the animals, inasmuch as they were pre-
viously unknown.

Four half-grown dilutes, possibly belonging to the same litter,
were seen in a waste-paper house during April. One of these,
of unrecognized sex, was reported by a workman as having been
caught in a trap. The second, a male, was brought in by the
workman; it has been dead for some time. Traps were set and
the third, a female, was caught alive on April 7, but died on
April 18. One female was found dead in the trap on April 11,
along with a wild-type male of the same size which was also dead.
Later captures from the same place are as follows:

On April 14, fourteen full-grown rats—three wild-type males,
ten wild-types females, and one dilute female. On April 15,
two adults—a wild-type female, and a dilute male.

On April 20, two adult wild-type females.

On April 25, one young wild-type female, possibly of the same
litter as the young dilutes.

On May 4, three of the wild type—one young male, one young

female, and one adult female; and three adult dilutes—two
males and one female.

On May 13, one adult wild-type male.

In all, nine dilutes and twenty-two of the wild type were cap-
tured in the waste-paper house.

Fifteen other wild rats were trapped during this time a short
distance from the waste-paper house, but none of them were
dilutes. For several years rats have been caught in large num-
bers in the near vicinity for study by investigators at The Wistar
Institute and at the Zoological Laboratory of the University of
Pennsylvania, but in no case were there any dilutes. It is very
probable, then, that the mutation occurred not long before the
dilute segregates appeared. It is also probable that some de-
gree of inbreeding must exist among wild rats, since the dilutes
seemed to be restricted to the waste-paper house and were there
in relatively large proportion. Dilutes were reported to have
been seen about the waste-paper house during the following sum-
mer, and one of them, a young male, was caught in August and
kept for a while at the Zoological Laboratory.

The rats taken in the spring of 1916 at the waste-paper house
were kept at The Wistar Institute. The two adult dilute fe-
males failed to breed, although paired with dilute males for some
six months. The three adult males were crossed with a number
of females of different colors. An attempt was made also to
breed from the wild-type rats taken in the waste-paper house by
crossing them with Albinos. Of the twelve wild-type females
tested only one produced offspring, and she gave dilutes as well
as wild-type young in the first generation. Five wild-type males
sired litters by Albino mothers, but only one of these had dilute
offspring. Thus of wild-type rats that bred two out of seven
carried the factor for dilution.

EXPERIMENTAL DATA

Dilute males crossed with black-hooded females sired seven
litters consisting of fifty-three individuals, twenty-seven males
and twenty-six females. All of these rats were gray Irish, show-
ing that the new form of dilution is completely recessive and that

the dilute rats carry the agouti and the self factors. Seven litters of F_2 rats were obtained, which contained forty-seven individuals, twenty-three males and twenty-four females. Of these individuals eight males and ten females were of the wild gray type; two males and three females were black; six males and three females were dilute gray; two males and three females were gray hooded; three males and two females were black hooded; one male and two females were dilute gray hooded, while one male and one female were dilute black or light sepia. The fact that hooding and non-agouti appeared among the dilutes shows that dilution segregates independently of these characters. There were in all thirty-three intense and fourteen dilutes, which is close to the expected ratio.

Dilute males crossed with albino females sired twenty litters consisting of one hundred and thirty-one individuals, seventy-three males and fifty-eight females. These were all distinctly lighter in color than the wild dilutes and appeared to be intermediate between ruby-eyed dilutes and albinos. We have called them fawn-colored. The fact that the cross with the Albino has failed to reconstitute the wild type indicates that the new dilution factor is allelomorphic with albinism, except for the improbable assumption that simplex albinism has reversed the dominance of ruby-eyed dilution. If the latter were the case, we should expect the F_2 generation to show some intensely pigmented individuals by recombination of factors, but as none of these appeared it is evident that the new dilution factor is allelomorphic with albinism. The Albino females used in this experiment were homozygous for black, or non-agouti, and for hooding. Wright ('17 c) has discussed the new dilution factor. We may follow his nomenclature and call self S and hooding S_h; agouti A and black a; intense pigmentation C_r, ruby-eyed dilution C_r, and albinism C_a. Thus expressed, the cross is dilute gray SS. $AA. C_rC_r \times$ Albino $S_hS_h.a\ a.C_aC_a =$ all fawn-colored $SS_hAa.C_rC_a$. The expected proportion in the F_2 generation would be nine dilute grays, three dilute blacks or light sepias, three dilute gray hooded, one light sepia hooded, twenty-four fawns (agouti and non-agouti), eight fawn hooded, and sixteen Albinos. In sixteen lit-

ters containing ninety-six individuals, fifty-eight males and thirty-eight females, there were: fourteen dilute grays, six males and eight females; two light sepias, one male and one female; seven dilute gray hooded, five males and two females; forty-two fawns, twenty-six males and sixteen females; nine fawn hooded, seven males and two females; twenty-two Albinos; thirteen males and nine females. In all there were twenty-three C_rC_r, fifty-one C_rC_a, and twenty-two C_aC_a, which is very near to the theoretical 1:2:1 ratio.

A wild-type male taken at the paper house was crossed with Albino females. Seven litters were produced containing fifty-nine individuals, twenty-seven males and thirty-two females. There were twenty-eight of the wild type, fourteen males and fourteen females, and thirty-one fawns, thirteen males and eighteen females. The fawns were inbred. They produced twenty-two litters consisting of one hundred and sixty-two individuals, eighty-nine males and seventy-three females. There were twenty-three dilute grays, seventeen males and six females; six light sepias, four males and two females; ten dilute gray hooded, five males and five females; three light sepia hooded, two males and one female; fifty-three fawns, twenty-two males and thirty-one females; twenty-four fawn hooded, fifteen males and nine females; forty-three Albinos, twenty-four males and nineteen females. This amounts to forty-two C_rC_r, seventy-seven C_rC_a, forty-three C_aC_a, which again is close to the expected ratio.

A wild gray female from the waste-paper house bred to an Albino male produced in two litters thirteen individuals, six males and seven females. There were of the wild type, four males and three females; and of fawns, two males and four females. The fawns inbred produced nine litters consisting of sixty individuals, thirty-six males and twenty-four females. There were seven dilute grays, four males and three females; two light sepias, one male and one female; four dilute gray hooded, two males and two females; two light sepia hooded, two males; twenty-two fawns, fifteen males and seven females; six fawn hooded, two males and four females; and seventeen Albinos, ten males and

seven females. This amounts to fifteen C_rC_r, twenty-eight C_rC_a, seventeen C_aC_a, which is also close to the expected ratio.

Summarizing the progenies recorded above of the wild gray male and female carrying the dilution factor, we find nine litters containing seventy-two individuals, thirty-three males and thirty-nine females. There were thirty-five of the wild type, eighteen males and seventeen females, and thirty-seven fawns, fifteen males and twenty-two females. The ratio of wild type to fawns is close to the expected 1:1 ratio. These fawns, when inbred, gave thirty-one litters consisting of two hundred and twenty-two individuals, one hundred and twenty-five males and ninety-seven females. There were thirty dilute grays, twenty-one males and nine females; eight light sepias, five males and three females; fourteen dilute gray hooded, seven males and seven females; five light sepia hooded, four males and one female; seventy-five fawns, thirty-seven males and thirty-eight females; thirty fawn hooded, seventeen males and thirteen females; and sixty Albinos, thirty-four males and twenty-six females. This amounts to fifty-seven C_rC_r, one hundred five C_rC_a, sixty C_aC_a, which is very near to the expected ratio. Finally we may summarize the progenies of all the fawns of the F_1 generations which were the offspring either of the wild gray male or female carrying the dilution factor crossed with Albinos or of the wild dilute males crossed with Albinos. There were in all forty-seven litters consisting of three hundred and eighteen individuals, one hundred and eighty-three males and one hundred and thirty-five females. In all cases the Albino parents were homozygous for black and hooding, $aa.S_hS_h$. C_aC_a. The fawns were then all $Aa.SS_h.C_rC_a$. The total F_2 generation is recorded in table 1.

The actual numbers are remarkably close to expectation. Summarizing for the albino series alone, we have eighty C_rC_r, one hundred fifty-six C_rC_a, and eighty-two C_aC_a—a close approximation to 79.5:159.0:79.5, the expected numbers.

Matings of F_2 dilute grays produced neither fawns nor Albinos, but did produce light sepias and hooded dilutes.

The wild ruby-eyed dilute males were crossed with red-eyed yellow females which had been sent to The Wistar Institute by

TABLE 1

COLOR	FORMULA	ACTUAL NUMBERS			EXPECTED NUMBERS	THEORETICAL RATIO
		♂	♀	Totals		
Dilute grays.........................	$A.S.C_rC_r$	27	17	44	44.72	9
Dilute blacks or light sepias.........	$a.S.C_rC_r$	6	4	10	14.90	3
Dilute gray hooded.................	$A.S_h.C_rC_r$	12	9	21	14.90	3
Light sepia hooded.................	$a.S_h.C_rC_r$	4	1	5	4.98	1
Fawns............................	A or $a.$ $S.C_rC_a$	63	54	117	119.25	24
Fawn hooded.....................	A or $a.$ $S_h.C_rC_a$	24	15	39	39.75	8
Albinos..........................	A or $a.$ S or $S_h.C_aC_a$	47	35	82	79.50	16
Totals...........................		183	135	318	318.00	64

Professor Castle. Red-eyed yellows along with pink-eyed yellows were obtained from England by Castle. Red-eyed yellow crossed with pink-eyed yellow has been shown to produce the black-eyed type, either agouti or black. The factor for red-eye, rr, shows partial coupling in both sexes with that for pink-eye, pp, according to Castle and Wright ('15). The red-eyed yellow females supplied by Castle were homozygous for self and for agouti. They did not carry pink-eye. When crossed to the new ruby-eyed dilute males they produced in all sixteen litters consisting of eighty-seven individuals, thirty-nine males and forty-eight females, all of the wild gray type. The reconstitution of the wild gray type in F_1 shows that the parental types are not determined by factors at homologous loci. The F_2 generation consisted of one hundred and one individuals, fifty-two males and forty-nine females. There were fifty-nine of the wild type, twenty-nine males and thirty females; nineteen ruby-eyed dilute grays, ten males and nine females; and twenty-three red-eyed yellows, thirteen males and ten females. On the basis of free interchange of the factor for ruby-eyed dilution with that for red-eyed yellow, we should expect 56.81 black eyes, 18.93 rubies, 18.93 reds and 6.31 ruby-reds. Castle ('16) has found indications that both red-eyed yellow and pink-eyed yellow, besides being

linked with each other, are linked with albinism. If this be the case we should expect to find them linked with ruby-eyed dilution. The failure of the double recessive, ruby-red, to appear in the F_2 generation is further corroboration of the linkage of the two loci.

The ruby-eyed dilutes have an eye-color distinctly lighter than that of the red-eyed yellows. The ruby-white compound, C_rC_a, which we have called fawn from the coat color, has an eye-color still lighter. A comparison of eye-colors in the rat has shown a gradation from black to pink as follows: black, $PP. RR. CC.$; red, $PP. rr. CC.$; ruby, $PP. RR. C_rC_r$; light ruby, $PP. RR. C_rC_a.$; pink, $pp. RR. CC.$; light pink, $PP. RR. C_aC_a.$ Other combinations may show intermediate grades.

The coat-color of the ruby-eyed dilutes is a very light sepia showing more or less yellowish white. The yellow tinge is probably due to excretions from the skin, since there are apparently no yellow granules. Albinos and hooded rats also show this yellow tinge in the hair, especially as they grow older.

Wright ('15) has shown quadruple allelomorphs in the albino series of guinea-pigs. He has further discussed the matter ('17 a) in a general scheme of color inheritance for mammals. A comparison of conditions in the rat with conditions in the guinea-pig will be of interest.

In the wild Norway rat the hairs are white at the base. Very gradually black pigment granules appear toward the tip and in all of the larger hairs these increase without interruption until the hair becomes intense black. In the hairs of intermediate and small size, however, the black pigment usually gives place to yellow granules which extend almost the entire length. The extreme tip is black. Some of the smaller hairs have black pigment in a single uninterrupted row of granules and others are white. A few of the smaller yellow-banded hairs have the band rather narrow and close to the tip. In almost all cases, however, when the yellow band occurs, it is very wide. In the black rat most of the hairs are white at the base, becoming black or dark sepia toward the tip, while a few of the smaller hairs are white throughout their entire length.

In fully colored ticked guinea-pigs the hairs of the back are sepia at the base and black toward the tip except that a narrow yellow band occurs on almost every hair at the short distance from the tip. In blacks the hairs are similar except that the band is lacking. Thus the intense guinea-pig is darker than the intense rat, for the hairs of the latter are white at the base. As described by Wright, there is in the guinea-pig a gradual decrease in the amount of yellow pigment from the intense, CC, through the various dilute combinations, C_dC_d, C_dC_r, C_dC_a, until the red-eyed dilute, C_rC_r, shows no yellow at all. In this animal the hairs are dark sepia banded near their tips with white. The dilute carrying albinism, C_dC_a, has light sepia hairs banded with light cream. Wright explains the correlation of the lighter sepia with the presence of yellow by assuming that in the animal C_dC_a there is competition in the oxidation of chromogen between the compound black producer, enzyme I–II, and the yellow producer, enzyme I. In the animal C_rC_r the yellow producer has been removed and enzyme I–II is free to act on chromogen without competition. All of these grades of pigmentation in the guinea-pig are darker than the ruby-eyed dilute rats, as is also the most dilute combination obtained in the guinea-pig, the red-eyed carrying albinism, C_rC_a. In this animal the hairs are light sepia to the base and are ticked with white near the tip.

The ruby-eyed dilute gray rat, $A.C_rC_r$, has no yellow pigment, but the yellowish tinge, as seen in the white rat, is noticeable in the lighter parts of the hairs; the black pigment is reduced to a very light sepia and is confined to the tips of the hairs. In the ruby-eyed light sepias, $a.C_rC_r$, the pigment is likewise very dilute, but extends from the tips well down the hairs. The base, however, is without pigment, as in the black rat. Thus the agouti and non-agouti dilutes can be distinguished, not by the presence or absence of bands, as in the guinea-pig, but by the greater extent of the pigment in the non-agoutis. The coat-color of the fawns, C_rC_a, is extremely dilute, midway between that of the homozygous ruby-eyes and that of the Albinos.

The hairs of agouti red-eyed yellow rats, $A.rr$, are yellow and

white. The larger hairs are white, while those of intermediate and small size are white at the base and tip only. For the rest of their length they have large yellow pigment granules. In the non-agouti red-eyes, $aa.rr$, the hairs are almost entirely white except for the yellow stain similar to that which occurs in white rats. There may be a very slight amount of extremely dilute sepia pigment in some individuals.

The double recessive, ruby-red, $C_rC_r.rr$, expected in the F_2 generation from the cross of ruby-eyed dilute gray by red-eyed yellow should be practically white even in the presence of the agouti factor, for the Albino type of dilution found in the dilute gray eliminates yellow, while red-eyed yellow dilution eliminates sepia. It is also to be expected that the double recessive, ruby-pink, $C_rC_r.pp$, will have white hair.

SUMMARY

A new Mendelian variety of the Norway rat known as ruby-eyed dilute gray has been found near the Zoological Laboratory of the University of Pennsylvania. The hair is light sepia at the tip and grades to white at the base. The eye color is ruby.

The new variation is recessive to intense pigmentation. When the dilutes were crossed to black-hooded rats all the F_1 individuals were intense and the F_2 generation showed thirty-three intense and fourteen dilutes.

Ruby-eyed dilution is allelomorphic with albinism. The F_1 individuals, called fawns, are intermediate both in hair and in eye color. Fawns when bred together produced eighty ruby-eyed dilutes, one hundred and fifty-six fawns, and eighty Albinos.

Ruby-eyed dilutes crossed with red-eyed yellow rats produce rats of the wild type. The second generation shows evidence of linkage of the two factors, since double recessives did not appear.

No linkage is apparent with hooding or with non-agouti.

In the agouti dilutes the sepia pigment is restricted to the tips of the hairs. The non-agouti are more heavily pigmented.

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STUDIES ON CYTOLYSINS

I. SOME PRENATAL EFFECTS OF LENS ANTIBODIES

M. F. GUYER AND E. A. SMITH

From the Zoological Laboratory, University of Wisconsin

1. INTRODUCTION

Ever since the pioneer work of Buchner in 1889 and of Behring and Kitasato in 1890, there has been a lively interest in the subject of so-called immune sera. The last few years have brought forth a flood of literature on antitoxins, agglutinins, precipitins, bacteriolysins, hemolysins, cytotoxins or cytolysins, and opsonins—all the fruition of these earlier investigations.

Inasmuch as the first discoveries were concerned with certain bactericidal properties artificially produced in the sera of animals through the injection of various species of bacteria, and because of the tremendous importance of these facts in furthering our knowledge of infection and immunity, the field became at once the province of the bacteriologist and the pathologist. Following closely upon the heels of the earlier discoveries came a brilliant series of practical applications in diagnosis, prophylaxis, and therapeutics, until it is not to be wondered at that investigators became absorbed in the medical phases of the subject. It is clear, however, that the phenomena in question all have their broader biological aspects, and it seems time To see if the knowledge already gained in this field may not be utilized in a new attack upon certain fundamental biological problems.

The work already done on precipitins, in fact, shows how these methods may be applied. When rabbits are injected with several doses of serum prepared from horse-blood, for example, their blood develops a substance not present in the blood of untreated

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rabbits. If the serum containing this substance is mixed with horse-serum in vitro, a cloudiness results, due to the precipitation of part of the protein in the horse-serum. The newly engendered ingredient of the sensitized rabbit serum which precipitates the horse-protein is termed a *precipitin*. The material injected, in this case horse-serum, is spoken of as the *antigen*. Not only can precipitins be formed against the blood-serum of an alien species, but against a wide range of substances, such as bacterial products, milk, peptone, globulins, and various albumins.

It is this specificity against a distinctly alien albumin that renders the precipitin test one of ready application in the medico-legal differentiation of human blood and various human albuminous substances from those of other animals.

Not only is the precipitin test useful in discriminating between non-related species, but it may prove to be important in establishing the taxonomic position of new forms and in confirming or changing the classification of groups already known, since among closely related species the specificity of the reaction is not absolute. For instance, Nuttall, who made observation on some 500 different animals found that rabbit-serum highly sensitized with human blood-serum reacts, though in varying degree, with the blood of all mammalia; a less strong serum, besides reacting on human blood, also causes a precipitate in the blood of anthropoid apes (chimpanzee, orang-outang, gorilla) and in a less degree in the blood of other monkeys; whereas a weak serum reacts with human blood and produces only a slight cloudiness in the blood of the anthropoid apes. He found that the same quantitative differences exist in antisera specific for each of the large vertebrate classes, birds, reptiles, and amphibia. Thus by the precipitin test a differential scale of actual relationships can be established.

The degree of activity of sensitized sera is judged by the dilution in which they will react. A properly sensitized serum will give a distinct reaction in blood diluted 1000 times. There are records of reactions with blood diluted 20,000 times or even 50,000 times, while Ascoli, indeed, reports obtaining a specific

reaction with a serum sensitized to egg albumin upon mixing it with egg albumin diluted 1,000,000 times. The delicacy of such tests can be appreciated when one knows that ordinary chemical tests cease to give reactions in blood diluted 1000 times.

As long ago as 1895 Bordet found that the blood of guinea-pigs which had been repeatedly injected with the red corpuscles of the rabbit acquired peculiar properties. Serum prepared from these sensitized guinea-pigs, when placed in a test-tube with rabbits' blood, not only caused the agglutination of the red blood corpuscles, but even rapidly dissolved them. The serum of untreated guinea-pigs was incapable of doing this or did it only feebly. Bordet showed further that this enhanced solvent action of the serum of animals treated with rabbit corpuscles existed only for the red cells of the rabbit, not for those of other species of animals. Exceptions to this rule have since been found, though in the main the action is a specific one. The action is known as *hemolysis*, and the substance in the serum which brings about solution of the red cells is termed a *hemolysin*.

Hemolysins are now known to be special members of a general class of substances termed cytotoxins or cytolysins. For just as alien red blood cells lead to the production of hemolysins, so various other materials, as leucocytes, nervous tissue, spermatozoa, and crystalline lens, when injected into the blood of an unrelated species, will form lytic substances more or less specific for the antigen used in the sensitizing process. All the cytolytic sera so far studied have been found to be more or less hemolytic, and it is probable that none acts exclusively upon its own antigen. The important fact, for our purposes, is that although a particular cytolytic serum may affect some other tissues, it vigorously attacks the special tissue used as antigen.

The exact nature of antibodies, such as precipitins, cytolysins and others, or the manner in which they are engendered is not known. The blood is probably in the main the carrier rather than the producer of such bodies. While the leucocytes may be one source of certain constituents, it is probable that various tissues of the body are responsible. There is evidence to show

that for some bacteria, at least, the bone-marrow, spleen, and lymph nodules produce the antibodies.

Although presumably distinct from one another, the various classes of antibodies seem to have many points of similarity, as, for instance, their method of origin, their reaction to heat, and their mode of operation. Chemically their natures are still unknown. Many of them seem to function through the combined action of at least two separate constituents. In some instances perhaps more constituents are involved. One of the components developed in response to injected foreign substances, such as bacteria, cells, dissolved proteins, various ferments, toxins, and venoms, though variously named by different workers, is commonly called the *immune body* or *amboceptor*. The other, an ingredient of normal serum, is termed the *complement*. Whether a single complement acts alone or a series of complements operate in conjunction with the amboceptor is a matter of dispute. Complement, in general, decomposes readily upon warming much above blood heat or spontaneously upon standing. It seems much less stable in every respect than the amboceptor, though the latter is the specific constituent developed through the introduction of an antigen.

The union of such foreign substances as amboceptors with a living cell is possible, according to Ehrlich's conception, because of the existence of a series of secondary chemical constituents or groups attached to the main living molecules of that cell. To these he has given the name of *receptors*. Since this term is in common usage, it is perhaps well to retain it until a better one for the existing condition is forthcoming, even though all workers would not subscribe to the implications it suggests. While receptors are supposed by Ehrlich to have a great variety of functions, he regards that of assimilation as of particular importance. To combine with these receptors any given substance (e.g., amboceptors) must possess, supposedly, very definite constitutional or configurational relations to the receptors in question.

One view looks upon the complement present in all serum as a sort of a ferment possessing digestive properties. It is power-

less until cells of the kind used as antigen have been rendered vulnerable by the action of the specifically generated immune body. Even the complement in an animal's own serum will suffice to dissolve one of its own tissues if the proper amboceptor is introduced.

Views as to just how these various elements combine and react are nearly as numerous as the investigators who have studied them. The explanation vouchsafed by the Ehrlich school for the lack of absolute specificity, is that certain of the different tissues of the body have receptors of the same kind, so that anyone of such a group of tissues might serve as an antigen for all the others. In other words, according to them, specificity is not a matter of cells, but of receptors.

There is urgent need for further investigation of cytolysins not only as regards this question of tissue specificity, but also as to the degree of specificity or lack of specificity for the homologous tissues on groups of related organisms. Furthermore, cytolysins have been developed so far for only a very limited number of tissue elements. The reports of pathologists who are seeking to develop specific cytolytic sera for cells of pathological origin, particularly malignant tumors, are increasingly discouraging, since apparently the distribution of common receptors is too widespread to permit the formation of antisera with sufficient specificity for medical purposes. But this difficulty need not block the biologist in his efforts to secure certain other results.

If it is possible to originate in living organisms antibodies which will destroy particular tissue elements, is it not possible to secure similar selective action on certain parts of the developing embryo? Moreover, if we are ever to break through the apparent impass which has enveloped our long-standing problem of the inheritance of somatic modifications, or that of provoking specific modifications in the germ through direct operation of external agencies, is not the employment of cytolysins possibly a line of attack which may yield fruitful returns? If a special serum will single out and destroy a certain element of the adult, is it not possible that there is sufficient constitutional identity between the mature substance of that

element and at least some of its material antecedents in the germ, that they too may be influenced specifically by the serum in question?

In an attempt to find answers to these and kindred questions, the authors of this paper have undertaken a series of experiments to produce specific antenatal effects in fetuses by means of cytolysins; to determine how early in an embryo cytolytic effects may be secured; to obtain cytolysins which will be operative in the developmental stages of certain periodically renewed structures, such as feathers; and particularly, to test the possibility of securing specific effects through the germ itself.

The present paper concerns itself mainly with the antenatal effects secured in rabbits and mice by means of fowl serum sensitized with lens. Experiments more or less similar to those here recorded are still in hand and others are soon to be undertaken on a more extensive scale.

2. EFFECTS OF LENS CYTOLYSINS ON FETAL RABBITS

The lenses of rabbits were used as antigen and chickens were employed as the source of the antibodies. The lenses, immediately upon removal from the dead animal, were pulped thoroughly in a mortar and diluted sufficiently with normal saline solution to permit of injection into the peritoneal cavity of the fowl by means of a hypodermic syringe. In order to prevent injury to internal organs, the fowl was held back downward that the viscera might settle away from the ventral abdominal wall, and the puncture was made just under the tip of the breast bone. At this point the needle enters easily, no internal resistance to the discharge of the liquid from the syringe is encountered and the fowl apparently suffers no pain.

When the fowl was ready for removal of the serum, killing proved to be more practicable than drawing off blood from the living bird. To secure the blood in sterile condition, the fowl was anaesthetized with ether until insensible, the feathers were hastily plucked from the neck and the latter washed in alcohol, the skin was cut entirely around the neck near the head and

peeled back, the oesophagus and trachea were transected and turned back so as not to discharge into the receptacle for catching blood, the head was then quickly severed near its base with heavy scissors and the neck thrust into a wide-mouthed sterile test-tube or flask.

Blood removed in this way was chilled in the dark for one or more hours and then centrifuged for some twenty minutes to separate the serum from the clot. The serum so obtained was, after warming to blood heat, injected into rabbits through the

TABLE 1

EXPERIMENT	DATE	FOWLS INJECTED	NUMBER OF RABBIT LENSES USED	NORMAL SALT SOLUTION	DOSAGE PER FOWL
				cc.	cc.
I........	November 1	1, 2, 3, 4	2	8	1.5
	November 8	1, 2, 3, 4	2	6	1.5
	November 15	1, 2, 3, 4	2	8	1.5
	November 22	1, 2 ,3, 4	2	8	1.5
III........	January 17	5–11	6	20	2.5
	January 19	5–11	6	20	2.5
	January 22	5–11	6	20	2.5
V*.......	March 8	12–16	4	20	2.5
	March 15	12–16	4	15	2.5
	March 17	12–16	4	15	2.5
	March 20	12–16	4	15	2.5

* All rabbits half grown.

marginal vein of the ear. All operations including those performed on rabbits were done with sterilized instruments and vessels and under as aseptic conditions as possible.

Experiment 1

In this experiment four fowls eight months old were used for sensitizing with lens material, and two pregnant rabbits, each of which had previously born normal litters, were the recipients of the sensitized fowl serum.

On November 1, 8, 15, and 22, respectively, the four fowls were injected intraperitoneally, each time with the lenses from an adult rabbit (table 1). The lenses were pulped and diluted with 6 to 8 cc. of

normal saline solution.   On each date each fowl received 1.5 cc. of
this mixture, containing approximately one-half of a lens.

On December 6 one of the fowls was killed and bled.   On December
7 the blood which in the meantime had been kept in the dark at a tem-
perature of about 5°C. was centrifuged and the decanted serum used
for injection into the two pregnant rabbits, each of which, between 10
and 11 a.m., was given 6 cc. of the. undiluted serum (table 2).   The in-
jection was made directly into the blood stream through the marginal
vein of the ear.   By 2.30 p.m. each rabbit showed evidence of illness,
particularly individual A, which was passing what appeared to be
bloody urine at frequent intervals.   Both had apparently recovered
by the next day.

December 9.   Both rabbits were given a second injection of serum
from another of the sensitized fowls which had been killed on De-
cember 8.   This time the dose for each rabbit was changed to 5 cc. of
serum diluted with 3 cc. of normal saline solution.   The animals
showed no ill effects after this treatment.

December 11.   Treatment similar to that of December 9 was given,
the serum being from the same source.

December 14.   Each rabbit was injected with 6 cc. of the undiluted
serum of a third one of the sensitized fowls, and this dose was repeated
on December 15, using serum from the same fowl.

December 18.   Each rabbit was given 6 cc. of undiluted serum from
the fourth fowl.

December 30.   Rabbit B, an Albino, gave birth to· seven young.
When the young finally opened their eyes, one had a slight opacity of
about one-half of the lens of the left eye.   This cleared up after three
days.   A second one had the entire left eye noticeably smaller, with the
lens opaque.   This opacity has persisted as has also the smaller size
of the eye.   At the present writing (October 15) this individual is
about fully grown.   While its right eye seems to be perfectly normal,
the left is but little larger than that of a newly born rabbit.   A third
individual had a cloudy rim around the edge of the lens which slowly
cleared after several days.

The four other young, in so far as one could judge from external
appearance, had normal lenses.   But since young of another mother
subjected to similar treatment had watery or liquefied lenses (see ex-
periment 5), a condition which was not detectable until the young were
killed and the eyes dissected, it is possible that cytolytic effects existed
in the lenses of some of these four apparently normal individuals.
One, in fact, when killed four months later (experiment 5) was found
to have a watery and diffuse lens.   By that time, unfortunately, two
of the other three had been disposed of so that it was impossible to
determine the consistency of their lenses.   The last one is being kept
for further breeding experiments.   She is at present mated with the
dwarfed-eyed one.

Rabbit A failed to bear young.   Since she is the one recorded as
passing what appeared to be bloody urine after the first injection, it is

TABLE 2

EXPERI-MENT	DATE OF INJECTION	NUMBER OF RABBIT	SE-RUM FROM FOWL	DOSE OF SERUM	REMARKS
I...	December 7	A and B	1	6 cc. serum	
	December 9	A and B	2	5 cc. serum + 3 cc. normal saline	December 30, B had 7 young
	December 11	A and B	2	5 cc. serum + 3 cc. normal saline	Visible externally in three individuals: 1 partly opaque lens in left eye (cleared up); 1 opaque lens in left eye (persisted); 1 cloudy rim (cleared up) May 19, 1 killed; watery lenses
	December 14	A and B	3	6 cc. serum	
	December 15	A and B	3	6 cc. serum	
	December 18	A and B	4	6 cc. serum	
III...	January 31	C and D	5	7 cc. serum + 3 normal saline	On February 15, rabbit C bore 5 young (all apparently normal, though 1 developed abnormal incisor teeth)
	February 3	C, D	6	10 cc. of a mixture of 8 cc. serum + 3 cc. normal saline	
	February 7	C, D	7	10 cc. of a mixture of 8 cc. serum + 3 cc. normal saline	On February 16, D bore 4 young (all apparently normal)
IV...	February 3	B	6	7 cc. serum + 3 cc. normal saline	March 6, B bore 6 young. Great discrepancy in size between individual members of litter
	February 7	B	7	8 cc. serum + 2 cc. normal saline	
	February 13	B	8	8 cc. serum + 2 cc. normal saline	
V....	March 31	E	12	10 cc. of mixture of 10 parts serum + 2 parts normal saline	April 24, 8 young born. Eyes weak and watery
	April 3	E	13	10 cc. of mixture of 10 parts serum + 2 parts normal saline	May 19, 1 killed and lenses found to be liquefied
	April 5	E	14	11 cc. of above mixture	

not impossible that she aborted at this time. She was remated successfully later and is the subject of experiment 2.

Experiment 2

Rabbit A of the former experiment was mated on January 10. She was not further injected with sensitized serum, as it was desired to find if the effects of the earlier injections might hold over and affect the young in utero. On February 12 she gave birth to eight young. None showed any perceptible effect of the lens cytolysin administered to the mother during the earlier experiment.

Experiment 3

In this experiment seven fowls were used for sensitization, and two pregnant rabbits, C and D. January 17, six lenses of adult rabbits were ground up in a mortar and diluted with 20 cc. of normal saline solution. Each of the seven fowls was given a 2.5 cc. dose of this suspension intraperitoneally. This treatment was repeated January 19 and again January 22.· Two of the fowls were bled January 30 in the usual way, and on January 31 the two rabbits, C and D, were each injected through the ear vein with 10 cc. of a mixture of serum 7 parts and normal saline solution 3 parts. On February 3 serum was taken from another of the sensitized fowls, and the two rabbits were each given a 10 cc. injection of a mixture of the serum 8 parts with 3 parts of normal saline solution. This was repeated on February 7 with serum from yet another treated fowl, using the same proportion of serum and normal saline solution as on February 3. On February 15 rabbit C bore five young. All were apparently normal at birth, though one rapidly developed abnormally large incisor teeth and had to be killed April 24 to prevent its slow starvation. The eyes of all were apparently normal. Rabbit D bore four young February 16, all with normal eye structures as far as could be judged from external appearances. The young of both of these experiments had been disposed of before the importance of determining the consistency of their lenses was realized.

Experiment 4

Rabbit B of experiment 1 was mated again January 24 to January 30. She was injected at intervals with serum from the sensitized fowls used in experiment 3, namely, on February 3 with a mixture of 7 cc. of serum and 3 of normal saline solution; on February 7 with a mixture of 8 cc. serum and 2 cc. of normal saline solution, and on February 13 with a similar dose. On March 6 she gave birth to six young which ranged in size from an extremely small one to one considerably larger than the others. No eye defects were visible. Again, because the eyes were of normal size and transparency, the young were disposed of, unfortunately, without determining the texture of the lenses.

Experiment 5

Five fowls were injected March 8, 15, 17, and 20, respectively, with lenses from young (about half-grown) rabbits, four lenses being used each time, so diluted with normal saline solution that each fowl received 2.5 cc. of the mixture. Rabbit E was injected in the usual way with serum from these fowls, the doses being:

March 31, 10 cc. of a mixture of 10 parts of serum and 2 parts of normal saline solution.

April 3, 10 cc. of a mixture of 10 parts of serum and 2 parts of normal saline solution.

April 5, 11 cc. of a mixture of 10 parts of serum with 2 parts of normal saline solution.

On April 24 a litter of eight young was born. All were tardy in getting their eyes open, one being particularly delayed in this respect. The eyes of all when opened seemed weak and watery. By May 17 all eyes seemed normal and most of the young were given away as pets in order to reduce the number of individuals which must be carried over the summer by a caretaker. On May 19 one remaining young one of this litter was killed. An examination of its eyes revealed the fact that its lenses were liquid, though still transparent. The lenses of three normal rabbits of the same age were examined and found to be fibrous and fairly firm. This suggested that the young in the litters of the other females which had been treated with serum might also have had lenses similarly affected though not visible from the exterior. Unfortunately, as already recorded, these had been disposed of before the discovery was made. One male of the litter produced in experiment 1 was still available. It was killed May 19, about four months after birth, and its lenses were found to be very watery and diffuse when compared with those of normal rabbits of the same age.

Since this same liquefaction of lens was found in the lenses of young mice in similar experiments which were being carried on by the senior author in California in the meantime (cf. section III), there seems no reason for doubting that it is an effect produced by a foreign serum sensitized to crystalline lens. A new series of experiments is being undertaken for further enlightenment on this point.

None of the adult females used in the foregoing experiments showed in the lenses of their own eyes any effects of the treatment. The tabulated data of the experiment are shown in tables 1 and 2.

3. EFFECTS OF LENS CYTOLYSINS ON FETAL MICE

The experiments on mice were performed by the senior author at the Scripps Institution for Biological Research at La Jolla, California. His thanks are due this institution for many courtesies. He is particularly indebted to Dr. F. B. Sumner and Mr. H. H. Collins for their generosity in adding to his stock of mice, for identification of species, and for information about breeding and rearing Peromyscus.

Chickens were used as the source of antibodies and lenses of Peromyscus maniculatus gambeli were employed as antigens. The fowls, averaging three and eight-tenths pounds in weight, were gradually sensitized, as specified in table 3, by repeatedly injecting emulsified lens intraperitoneally. To prepare this emulsion a number of lenses were ground up in a mortar in a little normal salt solution. When thoroughly pulped the mixture was thinned with more of the salt solution so that it could be readily injected by means of a hypodermic syringe. The proportions of lens and of saline solution in the various injections are specified in table 3. The individual fowls injected, ten in number, are arbitrarily designated by the letters A, B, C, etc., to J.

The purpose of the present experiments was to build up a lens cytolysin which when injected into pregnant mice (Peromyscus maniculatus gambeli) would have a solvent effect on the lenses of the young in utero. Judging from the results of earlier experiments on the rabbit, no effect on the lenses of the mothers was anticipated.

Precipitin tests

Inasmuch as there is no visible way to tell when serum is adequately sensitized for use as a cytolysin beyond trying it out directly, and since both time and the supply of pregnant females were limited, a series of lens precipitin tests were made with various of the fowls after the fifth and sixth injections, respectively in order to be sure that they were responding to the lens proteins. While there is possibly no necessary connection be-

tween the precipitin and the cytolysin reactions of the blood, it was felt that if the lens had so sensitized the fowls that precipitins were formed, one might infer that cytolysins had also been generated.

When ready to make these precipitin tests, several other species of Peromyscus were available from the experimenter's own and from Dr. Sumner's collections, hence it was possible to make a series of comparative tests. These are set down in full in table 4 because of the rather interesting relationships indicated. Since the tests were merely incidental to the other work, they are to be looked upon as suggestive rather than as

TABLE 3

DATE	FOWLS INJECTED	NUMBER OF MOUSE LENSES USED	NORMAL SALT SOLUTION	DOSAGE PER FOWL
			cc.	cc.
March 24...	5 (A, B, C, D, E)	26	15	3
March 31...	9 (A, B, C, D and F to J. E died)	42	27	3
April 7.....	9 (as above)	42	27	3
April 10....	9 (as above)	42	27	3
April 13.....	9 (as above).	96	45	5
April 24.....	8 (A, B, C, D, F, G, H, I, J died)	50	28	3.5

finished experiments. Judging from the results of these few tests, which are in harmony with the well-known work done several years ago by Nuttall,[1] it might be well worth some one's time to choose a genus such as Peromyscus and make extensive and accurate precipitin tests of various kinds on the different species with the view of finding physiological relationships and determining how these correspond to present taxonomic grouping and to geographical distribution. Interesting disclosures regarding conditions in hybrids should also come to light. For such work one should have narrow, very carefully graduated centrifugal tubes by which the amounts of precipitation could be accurately measured.

[1] Blood immunity and blood relationship, Cambridge University Press.

In making the precipitin tests the lenses were taken from four individuals of each species of mice to be tested, ground up in a mortar and diluted with 3 cc. of normal salt solution. The mixture was then filtered and to the resulting fluid 15 drops of fowl serum, sensitized as in table 3 with lens of Peromyscus maniculatus gambeli, were added. In the case of Peromyscus californicus insignis, which is much larger than other members of

TABLE 4

SPECIES OF MOUSE TESTED	PRECIPITATION AFTER EIGHTEEN HOURS

Serum of Fowl A.   April 30

1. Peromyscus maniculatus gambeli	Abundant
2. Peromyscus californicus insignis	About one-third that of 1
3. Peromyscus eremicus fraterculus	About one-third that of 1
4. Reithrodontomys megalotis longicauda	Very slight clouding
5. Perognathus fallax fallax	None

Serum of Fowl B.   May 4

1. Peromyscus maniculatus gambeli	Abundant
2. Peromyscus californicus insignis	Nearly one-half that of 1
3. Peromyscus eremicus fraterculus	Very slightly more than that of 2
4. Perognathus fallax fallax	None

Serum of Fowl C.   May 9

1. Peromyscus maniculatus gambeli	Abundant
2. Peromyscus eremicus fraterculus	About one-half that of 1
3. Peromyscus maniculatus sonoriensis	Almost as abundant as 1
4. Peromyscus maniculatus rubidus	Very slightly less than 3
5. Perognathus fallax fallax	None

the genus, four young individuals of about the size of adult P. gambeli were selected, because it was desired to have the amount of lens material used in each species as nearly the same as possible. After eighteen hours any precipitate that had appeared was concentrated in a centrifuge in order to get a more accurate estimate of the relative quantities formed.

It will be observed from table 4 that all species of the genus Peromyscus gave positive precipitin tests to serum sensitized

with lens of Peromyscus maniculatus gambeli, though the two subspecies of maniculatus (sonoriensis and rubidus) stood much closer to gambeli than did any of the others. This bears out the relationships as established by taxonomists. Reithrodontomys megalotis longicauda, the long-tailed harvest-mouse, which belongs to the same family as Peromyscus, gave a slight reaction, while Perognathus fallax fallax, the short-eared pocket-mouse, of an entirely different family, gave only negative results.

Effects of antibodies on the fetus

In attempting to get cytolytic effects on the young *in utero*, sixteen mice which had previously been mated for the purpose or which were obviously pregnant were used. Of these five proved

TABLE 5

MOUSE NUMBER	DATE OF INJECTION	SERUM FROM FOWL	REMARKS
1 to 9 inclusive	May 1	A	Nos. 1 and 2 died. No. 3 had 3 young May 2. No. 4 had 3 young May 4
5 to 9	May 4	B	No. 5 had 2 young May 6
6 to 9	May 9	C	
6 to 9	May 18	C	Nos. 6, 7, 8, dead, May 19. No. 9 died May 20
10, 11 (controls)	May 1, 4	Normal	No. 10 died. No. 11 had 4 young May 6

not to be pregnant, leaving eleven for the test. Two of these, used as controls, were injected with serum from a normal, non-sensitized fowl, so that nine were available for injection with the cytolytic serum. It will be seen from table 5 that the mortality was heavy and that only females which were well advanced in pregnancy and which, therefore, got but one or two doses of serum, brought forth their young. At each treatment every female was injected with 1 cc. of the sensitized serum.

Species of Peromyscus maniculatus gambeli only were used. The period of gestation in this form is twenty-one days and the young do not open their eyes until about sixteen days after birth.

It will be seen from table 5 that only three (Nos. 3, 4, and 5) of the females injected with the sensitized serum survived and bore young. Of these, No. 3 lost one of her young a few days

after birth, No. 4 ate one of hers, and No. 5 lost one of hers at
the end of the first week.   Thus, only five young of the females
treated with sensitized serum survived long enough to get their
eyes open.

Viewed externally, the eyes of these five young appeared
normal.   All showed the usual heavy pigmentation and no de-
fects were apparent in the lenses.   On May 23 all were decapi-
tated, the eyes were removed immediately and the lenses care-
fully dissected out.   In this way abnormalities which were invisi-
ble in the living animals came to light.

One of the young of No. 3 had both lenses normal in consist-
ency and transparency, the other one had the left lens normal,
the right markedly clouded.   One of the young of No. 4 had both
lenses translucent, the other one had an opaque zone around the
periphery of the left lens and a relatively large, scar-like white
area in the right.   From table 5 it will be noted that parents 3
and 4 had received only one dose of the sensitized serum, and
that late in pregnancy.   The remaining young one, from a
mouse (No. 5) which had been given two doses of the serum
had both lenses affected, the left being opaque and abnormally
small, the right transparent but of a liquid instead of the nor-
mal fibrous consistency.   The entire eyeball in fact, which con-
tained the dwarfed lens was upon removal readily seen to be
smaller than the other eyeball, or than that of a normal mouse
of the same age.

The four young of the surviving injected control, No. 11, were
killed the same day and were found to have lenses of normal
size, texture, and transparency.   It would appear, therefore,
that unsensitized fowl serum has no perceptible effect on the
lenses of the unborn young.

As a further control, six normal young, two sixteen days old
with eyes just opened, and four fifteen days old with eyes not
yet open, were also killed and examined.   Their lenses were all
transparent and of fairly firm texture.

The lenses of the treated mothers were also carefully exam-
ined, but all had remained of normal consistency and trans-
parency.

4. CONCLUSIONS

It seems legitimate to infer from the foregoing experiments on rabbits and mice that lens tissue of such forms when injected into fowls excites the production of specific antibodies which may attack in utero the lenses of the young of the species used as antigen. This reaction is not invariable, however, since, so far as one can determine by direct observation, a majority or even all of the individuals of a litter may not be acted upon, or a given individual may be affected in only one eye. The reason for such uneven effects is not apparent. It occurs to one, at first thought, that possibly the placenta is impervious to such antibodies except as occasional rupture of placental blood-vessels might permit of direct mingling of fetal and maternal blood. But such an hypothesis does not account for the fact that only one eye of an individual may be affected. Moreover, it seems improbable that mere accident to the placenta would account for such closely similar results as were found in both mice and rabbits.

The liquefactions described would indicate a true cytolytic effect. Of the several proteins composing the lens, one is fibrous, and it is upon this that the sensitized serum seems to have operated. Whether or not it had also affected the other ingredients could not be determined.

Whether the clouding and opaquing which occurred in others of the lenses should be regarded as the result of a cytolysin or of a precipitin is problematical. Further experiments are necessary to clear up this point. The important fact is that such opacity can be induced by specific sera and that it may become permanent. The most striking case among the rabbits would indicate that if the opacity is attributable to a precipitin reaction, a cytolytic effect accompanied it. For in this instance the affected lens has remained so reduced in size that the whole eye has been markedly dwarfed.

In so far as the literature on cytolysins records positive results, it leads one to expect specific effects in the immediate animal injected. But as already noted, no such effects were

observable in any of the injected females. A possible explanation of the lack of effect on the mothers may be that, because of meager circulation of blood in the lenses of adults, the quantity of cytolytic serum which reaches a lens is insufficient to affect it. In the developing eye of the young, the circulation is probably much fuller. The lenses in such forms, moreover, are in the process of formation and are not the fibrous masses which exist in older animals. For these reasons the lenses of immature animals are probably more susceptible to cytolytic and kindred agents.

The fact of chief interest is that visible specific structural modifications can be engendered in the young *in utero* by means of specifically sensitized serum. The present paper is to be regarded as a report of progress in a more extensive series of experiments.

REACTIONS OF THE PROBOSCIS OF PLANARIA ALBISSIMA VEJDOVSKY

WILLIAM A. KEPNER AND ARNOLD RICH

From the University of Virginia

TEN FIGURES

CONTENTS

MATERIAL AND METHODS

The planaria upon which we worked is found in the brooks and spring runs of the mountains about the University of Virginia. Collections were made most conveniently by gathering Vaucheria masses, leaves and stones from the bed of these mountain streams. When such material has been kept in an aquarium jar of tap-water, the worms rise to the surface. We have had no difficulty in keeping these animals for a week or more in tap-water that is free from infusion masses.

The animals so collected were fixed in chrom-aceto-formalin and aceto-sublimate solutions. Golgi preparations werealso made, but these yielded us no information concerning the nervous system. Iron haematoxylin and Mallory's connective-tissue stain gave us our best results in studying the nervous system of the flatworm.

NERVOUS SYSTEM

The central nervous system of this triclad consists of a pair of dorsal ganglia, connected by a transverse commissure and of two ventral nerve-trunks which give off at more or less regular intervals lateral and mesial branches. We have been able to trace a pair of the mesial branches of the ventral nerve-trunks towards the base of the proboscis, so that there is good reason for believing that the proboscis has a definite connection with the central nervous system, such as Gamble ('01) describes for triclads in general. As early as 1897 R. Monti ('97) wrote of the ventral nervous system of Dendrocoeles, "J'interprète donc les cordons longitudinaux comme une chaîne ganglion-naire non encore différenciée." Our own histological studies of Planaria albissima show that the ventral nerves are not mere bundles of nerve-fibers, but present a series of ganglionic masses along their entire extent. Steiner ('98) found that these ganglia exercised local control, for isolated posterior portions of Planaria neapolitana moved under control of ganglia in these parts of the body. The mesial branch of each of the ventral nerve-trunks leaves the ganglion that lies near the base of the proboscis to enter the proboscis.

ANATOMY OF THE PROBOSCIS

The proboscis, when freed, is a slightly tapering, almost cylindrical tube, its posterior end being the wider. Near the anterior end—the fixed end under normal conditions—there is a muscular ring, which acts as a sphincter. Both the inner and the outer surfaces of this tubular organ are densely covered with cilia. The wall is highly muscular. A definite nerve-plexus has been describe and figured (Gamble, '01) for the proboscis of planarians. From what we have seen of the histology of this organ, the proboscis does not greatly differ from the description of the anatomy of the proboscis of other Planaria. We have left the detailed histological study of this organ for a later piece of work. It is important, however, to mention here the presence of numerous glandular ducts which

extend throughout the entire length of the proboscis and open by means of pores along the margin of the mouth. The cell-bodies of these unicellular glands lie within the mesenchyme ventral and both posterior and anterior to the base of the proboscis. It is to be noted, therefore, that when a proboscis has freed itself from the body proper, all of its reactions are carried on without the aid of the cell-bodies of its peculiar unicellular glands.

NORMAL FUNCTIONING OF THE PROBOSCIS

Normally, as is well known, the proboscis functions as a prehensile organ. When the normal proboscis is ingesting food it is extended and its oral end lies projecting well out beyond the mouth of the proboscis-sheath. The oral end, as it approaches food, opens and closes, operating as a grasping, funnel-shaped structure. In addition to this muscular play of the circular lip of the proboscis, there is a movement of the cilia which line its lumen. This ciliary activity aids in carrying food into the opening and closing mouth. A peristaltic wave arises behind the food thus carried into the mouth, and this wave next travels anteriorly, driving the food ahead of it against the closed sphincter of the proboscis. After a mass of food has been collected near the sphincter, the latter opens and delivers the food to the enteron. This reaction of the fixed proboscis to food is not to be observed frequently under laboratory conditions. We have seen such reactions only twice, while none of the ninety members of the class in general zoology saw it. Food is not, therefore, readily accepted by the uninjured animals when they are being observed in the small amount of water present as the specimens are studied under the compound microscope. We have, however, been able to feed them with ease when they are retained in watch-glasses containing tap-water, thus making it improbable that the tap-water was highly injurious to the specimens, as Walter ('08) found to be the case for the worms and tap-water with which he worked.

REACTIONS OF FREED PROBOSCIS

Our attention was first directed to the conduct of the proboscis of this planarian while a class in general zoology was studying the worm. It was observed, in many cases, by the members of one section of this class, that, while they were studying the specimens under supported cover-glasses, the animals had cast off their proboscides which were swimming about, oral ends first, by means of their external cilia and ingesting various solid objects. In one instance Mr. Geiger called the attention to the fact that the freed proboscis of his specimen had turned upon its own body and had 'eaten a hole right through it.'

This observation suggested those made by Leidy ('47) on a planarian which had more than one proboscis. He said,

If one of these animals be punctured or cut, one or more of the proboscides will be immediately protruded as if they existed under pressure, and will move about in all directions, appearing as if entirely without the control of the animal; or if one of the animals be crushed between two slips of glass so that the proboscides will be torn from their attachment, they move about involuntarily, always in a line forwards or towards the mouth, which they do by contracting the stomachal extremity towards the oral, the latter remaining fixed. In this progressive course they constantly contract and dilate; the mouth opens and any matter in the vicinity rushes in, when it is closed and the matter passes onwards, and by the alternate contraction and dilatation of different parts of the same tube, it is thrown backwards and forwards several times, and finally violently expelled at the torn extremity. . . . . In fact, these curious independent movements caused me at first to mistake the organs for viviparous young.[1]

Darwin (43) also recorded that the proboscis of a land planarian of Brazil lives long after the body has been destroyed by salt.

Bardeen ('01 a) described briefly the normal food reactions of Planaria, and shows that a decapitated specimen will not find food material in a dish, although such a specimen could "be made to eat if it were placed on its back on a slide in a small drop of water. Under the conditions mentioned, the pharynx is usually protruded, and will

[1] Mr. W. H. Taliafero called our attention to this reference and that which we have made from Walter ('08).

engulf bits of food placed in the mouth." An experiment was performed in which the part of the head in front of the eyes was cut off. Such specimens, from which merely the tip of the head had been removed, reacted normally to food. It is also shown that specimens from which the part of the body posterior to the pharynx has been removed feed like normal worms.[2]

We have observed that the reaction of the incomplete proboscis of Planaria albissima is variable. When it lacks more than the cell bodies of its basal glands, it shows but little departure from normal conduct—the three coördinated movements of food ingestion being carried out.

There is greater variability in the reaction of the proboscis when the sphincter at its base is removed, as the following observations indicate.
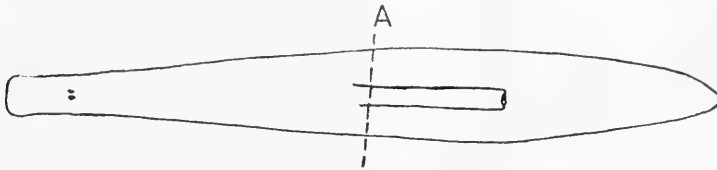

Fig. 1 *A*, transverse cut removing sphincter of proboscis; proboscis inactive except for ciliary movement.

A specimen was cut transversely at *A*, figure 1, so as to free a proboscis which lacked the sphincter. The reaction of the cilia of the external surface of this proboscis carried it from the proboscis sheath. The proboscis then lay for ten minutes by the side of a piece of planarian body and then, without showing any reaction to the food, slowly moved away. There were no opening and closing movements of the mouth on the part of this specimen and there were no peristaltic movements in the wall of the proboscis. In another experiment, after two parts had been removed from the body of the specimen by transverse cutting (fig. 2, *B* and *C*), a third cut was made so as to pass through the base of the proboscis (*D*). Here again the proboscis, less a sphincter, swam from the sheath by ciliary activity and then lay quite inactive. Next the proboscis was severed

[2] Quoted from Pearl ('03), p. 523.

as indicated at (*F*). Immediately the oral end opened and closed in an indifferent manner, so that food, which lay near the mouth, was not ingested. No peristaltic waves arose in the aboral part of the organ. Finally, in a third specimen (fig. 3), we had a case in which the freed proboscis was quite active when complete (*G*); but there were evident no longer any of the movements which play a rôle in the ingestion of food when the oral fourth of the proboscis was removed (*H*). A marked variation from the reaction of the above incomplete proboscis is
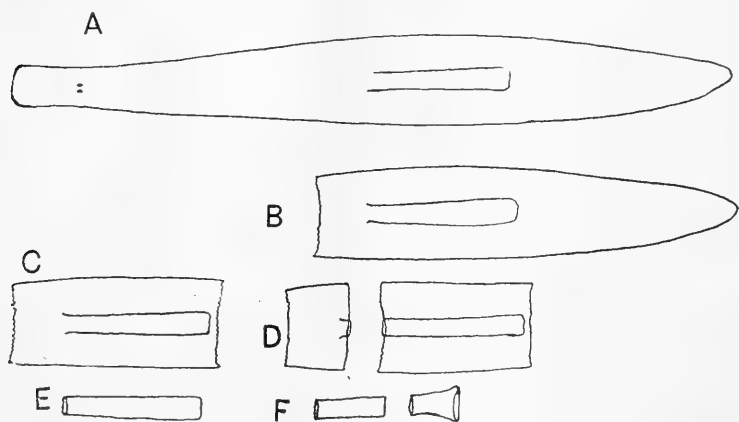

Fig. 2 *A*, entire animal; *B*, dorsal ganglia and anterior portion of body removed; *C*, posterior portion of body cut away; *D*, proboscis severed through base, sphincter removed, proboscis then swam from sheath and remained inactive; *E*, free proboscis; *F*, proboscis severed transversely, oral end opened and closed in an indifferent manner.

that of the specimen shown or represented in our figure 4. Here the sphincter (*D*) was cut away, and though the movements of the remainder of the proboscis were not normal (more spasmodic), still the proboscis mouth explored for food and ate. The larger part of this proboscis swam about, and in coming by its own detached sphincter accepted the latter by greedily ingesting it. Unless, therefore, the proboscis be complete enough to permit of all of the three coördinated movements being effected, no two and usually no one of this set of movements will be well carried out.

FACTORS DETERMINING INHIBITION OF THE PROBOSCIS

We have been interested most in an effort to determine what the inhibitory influences are which control the fixed proboscis in such manner that it rarely ingests objects under conditions
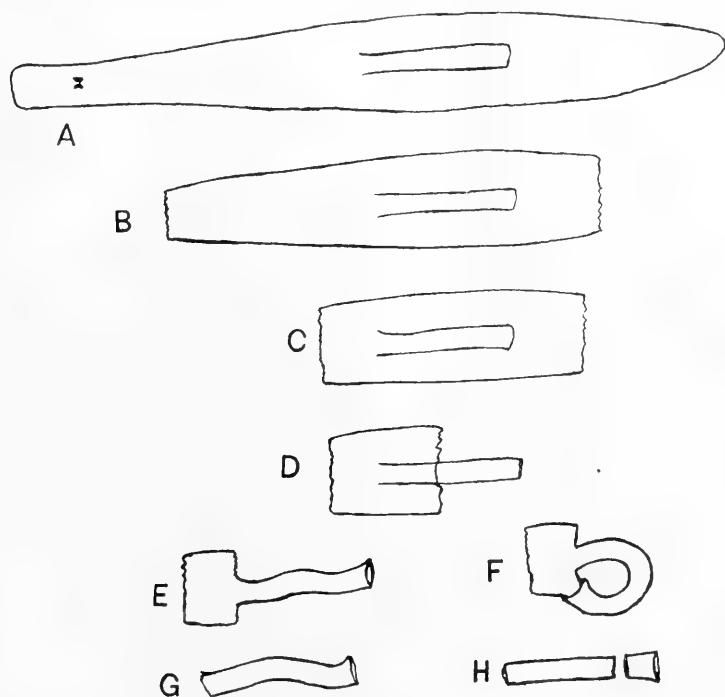
Fig. 3   *B*, dorsal ganglia and posterior extremity of body cut away; *C*, more of anterior portion of body removed; *D*, posterior half of proboscis-sheath cut away; *E*, entire proboscis-sheath removed, proboscis becoming active (*F*); *G*, proboscis active after autoamputation; *H*, proboscis inactive after oral fourth was cut away.

favorable to microscopic study, thus making the fixed proboscis stand in sharp contrast to the freed proboscis, which latter under laboratory conditions may readily be demonstrated ingesting food and other solid objects. It has occurred to us that one of these inhibitory factors may be external and the other internal.

The observations of the men of the class in general zoology suggested that perhaps a disturbance of thigmotactic conditions resulting from shallow water or pressure of the cover-glass were responsible for the autoamputation of the proboscis and that its reaction was not due to disorganization of the body or parts of the body. This suggestion is further supported by two observations made later by ourselves. On two occasions we had
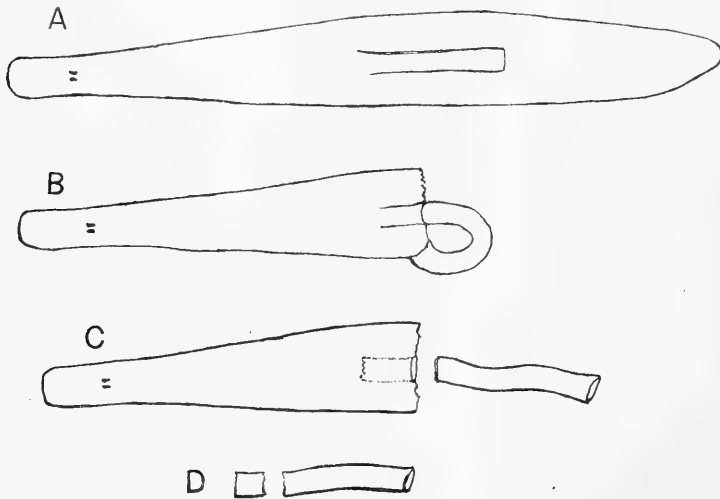
Fig. 4 *B*, two-thirds of proboscis-sheath and posterior portion of body cut away, proboscis active and ingesting body proper; *C*, proboscis active after auto-amputation; *D*, sphincter of proboscis removed.

drawn away the water from specimens, so that the worms had but a mere film of water over them. In these specimens, thus stranded in quiet shallow water, it was noticed that the proboscis had broken from the body in each case and was swimming or wriggling about within the sheath. To one specimen water was added as soon as the proboscis had freed itself. After the addition of this water the proboscis left the sheath and swam about in the water quite as actively as did the body proper from which it had been separated. In the other specimen the body proper, while in the shallow water, became so intimately

fixed to the slide that when water was added it appeared as a distorted mass which the freed proboscis was ingesting. More-over, the proboscis when inactive lies within a sheath, the walls of which lie more or less in contact with the quiet proboscis. It has been noted that, when the proboscis is extended to pro-ject from the mouth of the sheath, the part that lies free is the portion of the proboscis that is actively contracting and mov-ing. The part within the sheath shows little or no activity until food passes through it. Hence we tentatively inferred that ab-sence of contact with the walls of the proboscis-sheath excites the proboscis into activity.

The following experiments were made to determine whether the absence of thigmotactic stimuli excites the proboscis beyond the inhibitory control of the central nervous system.

1. A specimen had its anterior and posterior ends cut away (fig. 3, B). Next an additional part of the anterior end was am-putated. There was up to this step no reaction on the part of the proboscis (fig. 3, C). The posterior half of the proboscis-sheath was next cut away and as yet the proboscis remained quiet (fig. 3, D). When, however, all of the sheath was removed, al-though the proboscis remained joined to the fragment of body at its base and hence in connection with a part of the central nervous system, it became active at once, turning upon the frag-ment of body and ingesting part of it (fig. 3, F). Finally, the proboscis underwent autoamputation and swam about attempt-ing to ingest objects. This reaction and autoamputation of the proboscis may have been due, in this case, to a mechanical injury of the central nervous system.

2. In this second experiment we had the proboscis incited to activity under other conditions. Here, after the anterio and posterior ends had been removed (fig. 5, B), the proboscis left the sheath when an effort was made to pull away the sides of the proboscis-sheath with two needle points. While the sheath was thus temporarily spread the proboscis swam out. Here again there was a temporary disturbance of the thigmotactic condi-tions existing between the proboscis and its sheath; but the ques-tion of disturbing the central nervous system, during the opera-tions, also presents itself.

3. Finally, we have in our third experiment a case in which a posterior part of the body was amputated so as to leave at least two-thirds of the proboscis projecting free (fig. 4, *A*, *B*, *C*). Here the possibility of mechanical injury of the controlling portion of the central nervous system during the operation is much less, and yet the proboscis immediately became active and turned anteriorily in an effort to ingest part of the body to which it was attached. The proboscis in this specimen soon underwent autoamputation.

This last is a strikingly exceptional case and is the only example in which we have it suggested that thigmotactic stimuli, independent of nervous control, are factors in the inhibitory control
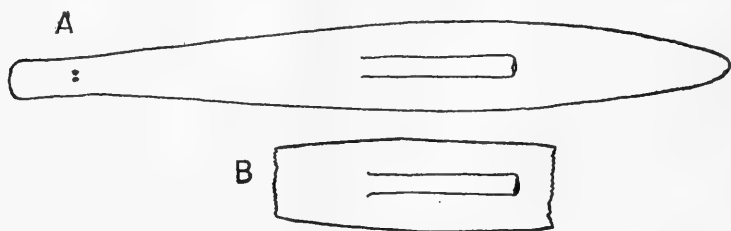
Fig. 5 *B*, anterior and posterior extremities removed. On attempting to open sheath with needle points, proboscis underwent autoamputation.

of the proboscis. Even here there may have resulted an injury of the ganglia or nerves of the proboscis, so that the breakdown of the inhibition was due to nervous injury and not to thigmotactic disturbances. We have, therefore, no clear case that shows that thigmotactic disturbances will alone excite the proboscis into abnormal activity.

Our experiments, however, indicate that the central nervous system acts as an inhibitor to the proboscis. Not all of the central nervous system seems to be directly concerned with this inhibitory control.

1. The dorsal ganglia of a specimen were amputated (fig. 6, *A*), and no marked disturbance of the proboscis followed. Ten minutes later a second cut was made midway between the base

of the proboscis and the first cut (*B*); three waves passed over the proboscis during five minutes, otherwise it was quiet. Then a cut was made quite near the base of the proboscis (*C*) and the latter writhed in the sheath for two minutes, then became inactive. The next cut was made at the base of the proboscis, and at once the proboscis became active and wriggled out of a tear in the sheath that had been made accidentally. After ninety seconds the proboscis suffered autoamputation (*D, E*).
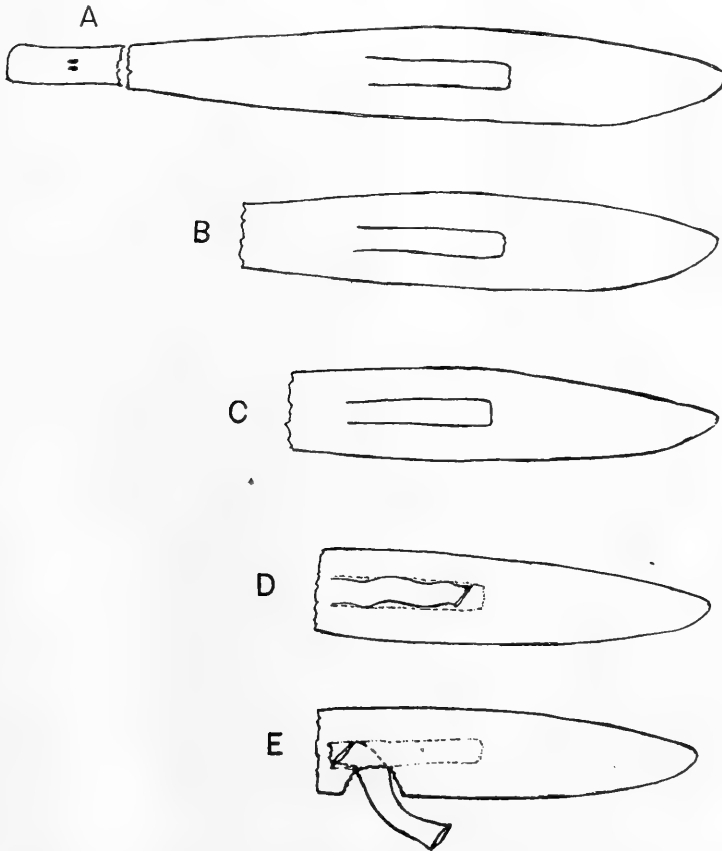
Fig. 6 Dorsal ganglia amputated; *B*, more anterior portion removed; *C*, additional removal of anterior part of body; *D*, cut made near proboscis base, proboscis in sheath; *E*, proboscis underwent autoamputation and wriggled from rent made accidentally in sheath.

2. A specimen was impaled upon a needle point near the right-hand margin of the proboscis. The animal, in freeing itself, made a tear in its body that passed obliquely from near base of proboscis-sheath posteriorily to right margin of body (fig. 7, *A*). The proboscis swung out from this rent and projected as a passive, quiet object. Next the sheath and posterior part of the body were torn away leaving the proboscis trail-
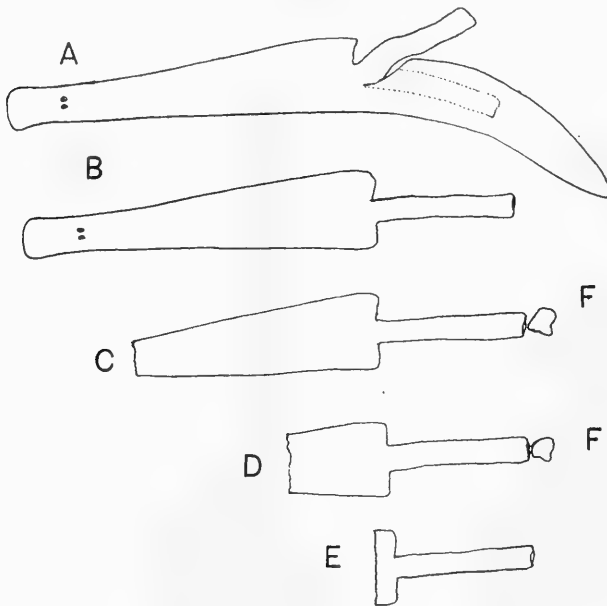
Fig. 7   *A*, rent made at right side of proboscis near its base, proboscis swung out, inactive; *B*, posterior portion of body removed, proboscis inactive; *C*, dorsal ganglia removed, proboscis inactive; not accepting food (*F*); *D*, further portion of anterior part of body removed, proboscis inactive, not accepting food (*F*); *E*, cut made near base of proboscis, proboscis active.

ing behind the swimming animal (*B*). In this condition the proboscis remained perfectly quiet. The dorsal ganglia were amputated and a piece of food pushed after the swimming animal near the mouth of proboscis, but as yet there was no reaction on the part of the proboscis (*C*). One-half or more of the remaining anterior portion of the body was cut off. After

this the specimen swam no longer and a piece of food was placed in contact with the oral end of the proboscis; still the proboscis remained quiet (D). Finally, when the portion of the body proper was reduced to a piece relatively as small as indicated in figure 7, E, the proboscis became active at once, swung to and
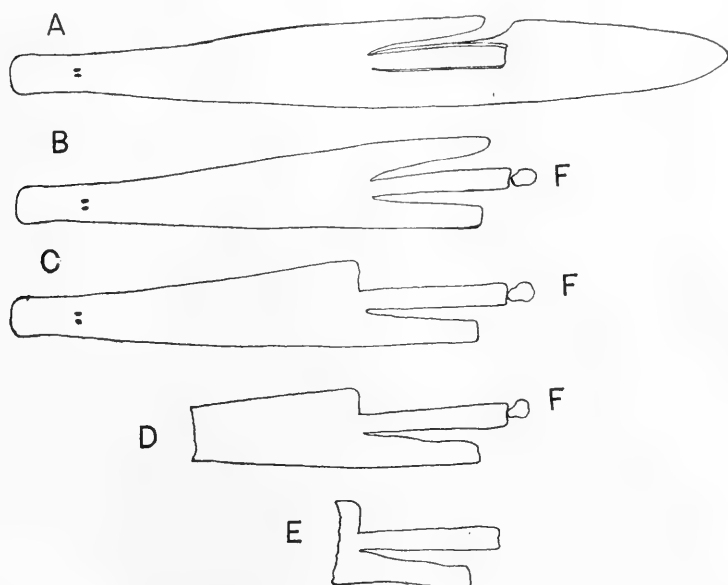
Fig. 8 A, rent made by animal tearing from needle point upon which it was impaled; B, similar rent made on other side, completely exposing proboscis, proboscis inactive, not accepting food (F); C, portion of one side of body cut away at proboscis base, proboscis yet inactive, not accepting food (F); D, dorsal ganglia amputated, proboscis inactive, not accepting food (F); E, cut near base of proboscis, proboscis active.

fro, and in a moment broke away and swam about ingesting fragments of its own body for more than seven minutes.

3. Figure 8 shows a rent made in a specimen by impaling it on the right side of the body near base of the proboscis and holding it on the needle until the animal tore from the needle. In a like manner a similar rent was made at the left side of the

body. When this second rent was made, the posterior part
of the body was torn away (*B*). In neither case when the rents
were being made did the proboscis show any reactions, though
food (*F*) was pushed after the projecting proboscis of *B*. The
projecting part of the body to right of the proboscis was ampu-
tated, and as yet no reaction of the proboscis followed (*C*). About
half of the remaining anterior portion of the body was next
removed. The specimen no longer swam and when food (*F*)
was placed near the oral end of the quiet proboscis, the latter
showed no response (*D*). Finally practically all of the body
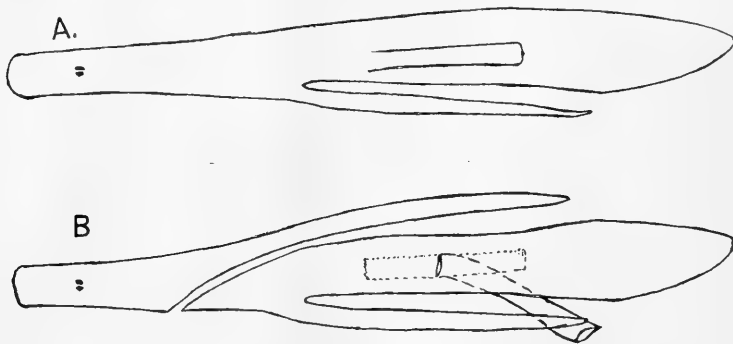
Fig. 9   *A*, cut made removing left lateral nerve-trunk from region of pro-
boscis, proboscis inactive; *B*, cut made removing dorsal ganglia and right lateral
nerve-trunk from region of proboscis, proboscis active.

proper was removed, as indicated by *E*, and immediately the
proboscis set up exploratory movements and a swinging to and
fro. Eventually the proboscis broke away and, as it swam
through the water, pushed a piece of its own body ahead of its
mouth.

Further evidence of the inhibitory influence of the central
nervous system is to be seen in experiments like the following,
in which the specimens were cut more or less longitudinally
instead of transversely.

4. In specimen shown in figure 9 a lateral cut was made
with a razor, which severed the connection between the proboscis

and the ventral nerve of that side of the body. There was no evidence of reaction of the proboscis. Then the other margin of the body with its ventral nerve was cut away, and, though there was no damage done to the sheath or to the base as such, the proboscis at once suffered autoamputation and swam about ingesting food (*B*).
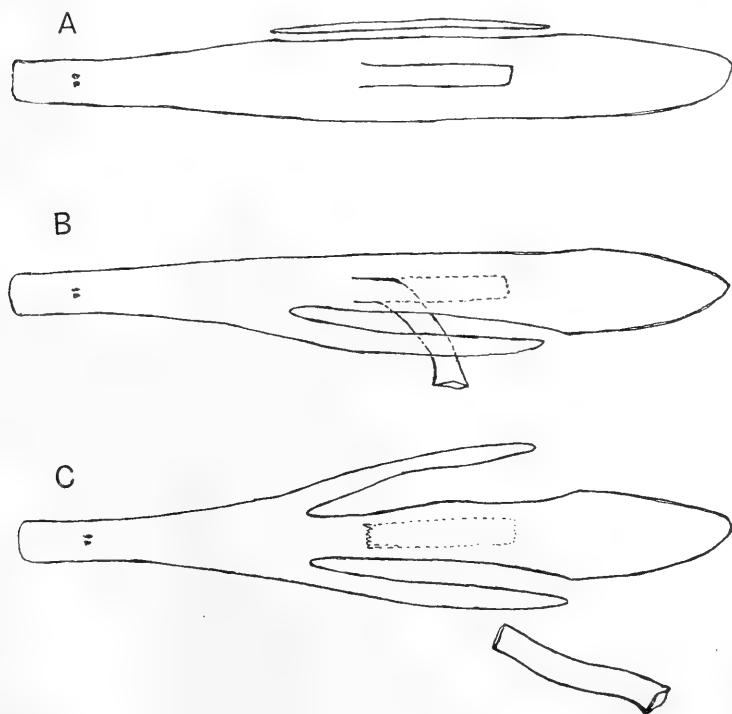
F g. 10   *A*, cut made on right side of body too shallow to include lateral nerve-trunk, proboscis inactive; *B*, cut on left side removing lateral nerve-trunk, proboscis projected, but remained inactive; *C*, second cut made on right side removing other lateral nerve-trunk, proboscis active.

5. Figure 10 shows an experiment that yields evidence of this nervous inhibition. First a very narrow edge of the body was cut away—too narrow to include the nerve-trunk. There was no reaction of the proboscis. Then, from the other side a strip was cut deep enough to take away the nerve-trunk of that side.

The proboscis projected itself from the sheath, but hung there perfectly quiet, showing no signs of activity. After several minutes a second strip was cut more deeply from the side from which the first narrow slice had been taken. This second cut was deep enough to remove the nerve-trunk of that side, and the proboscis at once underwent autoamputation and became active. It is thus indicated that the severing of connection between the proboscis and both lateral nerve-trunks is necessary, when the sheath is intact, for the removal of inhibition sufficiently to cause the proboscis to become hyperactive.

The manner in which the basal ganglia have been removed in our experimenting is rather crude. Needles, scalpels, and razors were used in this work. It is quite possible that in these operations not only were the nerve connections destroyed, but pressure in a varying degree must have been brought upon the proboscis-sheath. This pressure as it varied might have disturbed the thigmotactic conditions within the proboscis-sheath. This latter contingency may account for certain variations in the reaction of the proboscis as observed by us. For example, two of three animals that had been severed but once, and that near the bases of their proboscides, underwent autoamputation of their proboscides and had their prehensile organs leave their sheaths. The proboscis of the third specimen wriggled actively within its sheath, but only swam out of the sheath when a hole was made in the sheath with a needle. In addition to this variation of reaction on the part of the proboscis, each of us has had cases in which, after the ganglia adjacent to the base of the proboscis had been removed, no autoamputation resulted. More experimentation is needed to determine the cause of this variation. At present we can only suggest that in cases like these last ones we had caused no disturbance of thigmotactic conditions in the sheath when we were removing the ganglia, hence the proboscis was not excited and showed no response, though the inhibition of the nervous system had been destroyed.

It is indicated by the above experiments that the removal of the ganglia posterior to the base of the proboscis does not materially disturb the control of the organ. The amputation of

the more anterior portions of the central nervous system may result in slight disturbance of the proboscis, while the ganglia adjacent and anterior to the base of the proboscis ordinarily act as inhibitors to the ingesting reflexes of the proboscis.

REACTION TO FOOD OF FREED PROBOSCIS

The ability of the proboscis that had undergone autoamputation to distinguish between food and non-food was tested in the following manner: After a proboscis had separated from the body it was washed in several changes of fresh tap-water to free it from any solutions or particles of its own body, than might serve as a stimulating agency to the proboscis. Fragments of washed cover-glass were placed into clean water that contained such a washed proboscis. In some instances these fragments of glass were accepted by the proboscis and were passed by the sphincter and thrown from the organ as food is handled by free proboscides. The ingesting of objects by a freed proboscis displays no choice, hence it is a reflex.

CONCLUSIONS

1. All proboscides of Planaria albissima that have been severed from their adjacent ganglia show some reaction by disturbed movements within the proboscis-sheath. Most of the proboscides thus separated from the central nervous system underwent autoamputation while lying within the sheath. In a relatively few instances we have found that, unless the proboscides in addition to being cut off from the central nervous system have been excited by a disturbing of the thigmotactic conditions within the sheaths, they do not undergo autoamputation. *In all cases, however, the disturbance of the thigmotactic conditions of the sheath so excites the proboscis that, without the inhibitory control of the adjacent ganglia of the central nervous system, the proboscis suffers autoamputation and acts as an independent reflex organism.*

2. The freed proboscis is able to carry out the three coordinated muscular movements involved in the mechanics of

food ingestion, but this only when the entire musculature of the proboscis is intact.   The freed proboscis had not the ability to distinguish between food and non-food.   The exercise of choice is made possible only through the functioning of the central nervous system.

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ON SEVERAL EFFECTS OF FEEDING SMALL QUANTI-
TIES OF SUDAN III TO YOUNG ALBINO RATS[1]

S. HATAI

From The Wistar Institute of Anatomy and Biology

THREE CHARTS

Daddi's ('96) observations on rabbits, guinea-pigs, and fowls which showed that Sudan III mixed in oil is readily absorbed from the alimentary tract, and is ultimately deposited in the adipose tissue, induced numerous investigators to study the problem of the metabolism of fat by the use of this dyestuff. Recently Ehrlich and his pupils demonstrated that certain dyestuffs can stain some micro-organisms in vivo, and indeed in some instances such chemical substances thus introduced into the animal body produce a curative effect. This new line of investigation, now called 'chemo-therapy,' induced numerous investigators to examine the possible affinities between organisms and various dyestuffs, including Sudan III, and consequently the literature on the feeding of Sudan III is quite large. I shall therefore review only a few studies which are intimately concerned with my own experiments.

Riddle ('08) fed Sudan III to hens during the laying period, and demonstrated that this dyestuff is easily absorbed into the egg and the yolk fat intensely stained.

In another paper, Riddle ('10) gives further observations on feeding Sudan III to domestic fowls, and not only extends his

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[1] This research was closed and set aside in January, 1916, because it had not given the information originally sought, and the further study of the atrophy of the thymus and other viscera did not fall within our program. The problem of the changes in the thymus has now been taken up by Dr. Ivan Wallin, and in view of the fact that the work is to be carried on, it has seemed proper to publish the results already in hand.—S. H.

earlier observations on the deposition of Sudan III in ova as well as in the soma, but reports also his incidental observations on the growth of the chick fed with Sudan III, as well as those on the question of fat metabolism.   In brief, he finds that Sudan III tends to retard the growth of the chick.   He also notices the production of defective feathers among these test chickens.

Gage ('08) repeated Riddle's experiment and found that while Sudan III is deposited in the hen's egg, it does not appear in new-born albino rats whose mother has been fed with this dye-stuff for some days previously.   Gage thinks that the placenta prevents the entrance of Sudan III into the fetal circulation.

In 1912 Mendel and Daniels published their extensive observations on fat metabolism, using not only Sudan III, but several other fat-soluble dyestuffs.   These investigators touch on numerous interesting problems regarding the fate of the dyes after these are taken into the animal body, and I shall later on refer to this important paper.

Another study was made by Corper ('12), who fed guinea-pigs with several dyestuffs which are soluble in fat, including Sudan III, with a view to studying the biochemistry and chemo-therapy of tuberculosis.   Corper fed the animal with a rather large dose (0.025 grams per diem) of Sudan III for long periods (over 200 days) without apparently producing any ill effects.

While I was feeding Sudan III to young albino rats to determine whether it appeared in the newly formed myelin sheaths, I noted that the rats which were receiving a small amount (about 8 to 9 milligrams per diem) of Sudan III mixed in olive oil, not only failed to grow, but appeared in many respects strikingly abnormal.

Preliminary examination of the organs revealed the fact that most of these organs were distinctly altered in appearance.   The most noticeable alteration was the complete atrophy of the thymus in every one of these seven test rats.   Inasmuch as these alterations were not recorded by previous investigators, and particularly because of the behavior of the thymus towards Sudan III, the present investigation was undertaken.

METHOD OF INVESTIGATION

For this investigation albino rats alone were used. The rats were from 27 to 33 days of age and were still running with mother. The litter was divided into two groups; one group, the controls, was fed with Austin's dog biscuit, and the other, the tests, received approximately 0.008 to 0.020 milligrams of Sudan III (Grübler's) in the form of a solution in olive oil well mixed with about 5 grams of the powdered dog or rat biscuit. The amount of oil[2] used was about 1 to 2 cc. per rat per day. When 20 milligrams of Sudan III were given the dose was found to be too strong for the young rats to withstand for more than one week, and indeed at the end of one week the test rats were so emaciated and sick that it was necessary to return the animals to the normal diet for recovery.

Altogether 135 rats belonging to 19 litters were used, and from the study of them I can present a few examples of the growth of the body and organs of rats fed with Sudan III mixed in olive oil. In each series the controls and the test animals were always from the same litter.

GROWTH OF BODY IN WEIGHT

Table 1 gives the growth record for five of the series. These are typical. The corresponding graphs for the growth of the body in weight as the result of Sudan III feeding are shown in chart 1 for series I, II and III and chart 2, for series VIII. Both charts show clearly that the rats fed with Sudan III do not grow as well as their litter mates of the control group. The amount of Sudan III administered was small (8 milligrams), nevertheless it is sufficient either to inhibit the growth entirely or retard it to a considerable extent. In general the younger rats appear more sensitive to Sudan III than the older. In fact it has been found that the rats with body weights of more than 50 grams show a high resisting power to this dyestuff, and it requires a considerably longer period to produce marked results.

[2] The oil used in all of these tests carried the trade-mark: Extra fine—James Wagner (Philadelphia)—Made in France. It was not tested chemically.

　　　　　S. HATAI

TABLE 1

Showing the growth in body weight of albino rats fed with Sudan III dissolved in olive oil, contrasted with the control rats fed with the normal laboratory diet. In series X the control rats were fed with the laboratory diet plus olive oil alone.

BODY WEIGHT GRAMS	SERIES I, II, AND III						SERIES VII.		SERIES X	
	Control			Sudan III and oil dose 8-9 mgms.			Control	Sudan III and oil, dose 10-15 mgms.	Control (oil)	Sudan III and oil dose 8-9 mgms.
	♀ I	♀ II	♂ III	♀ I	♀ II	♂ III	2♀+1♂	2♀+1♂	1♀+2♂	1♀+2♂
Days after Sudan III feeding										
Initial	30.0	32.1	44.4	30.5	35.6	41.9	28.7	29.7	32.4	32.7
7	46.8	41.9	58.5	34.6	36.2	42.1	35.7	29.6	38.1	33.7
10							40.3	29.2	41.3	34.8
14	68.4	58.9	75.0	38.9	39.0	50.0	46.9	30.1	48.0	35.9
17							51.1	30.4	52.5	36.8
21	86.5	70.7	90.0	40.4	40.4	55.5	57.6	31.1	59.2	37.4
25	93.1	75.0	97.5	41.3	42.5	54.0	62.1	30.4	61.4	36.1
28	97.8	80.9	103.4	42.3	42.0	55.0	68.4	29.6	69.1	36.4
31	106.4	87.8	111.0	42.8	41.5	51.5				
35	110.9	90.2	114 5	42 0	41.5	50.0				

Series　　I 28 days old at the beginning of the experiment.
Series　　II 29 days old at the beginning of the experiment.
Series　　III 33 days old at the beginning of the experiment.
Series VIII 27 days old at the beginning of the experiment.
Series　　X 28 days old at the beginning of the experiment.

Riddle found that chicks do not grow well on a Sudan III diet, and furthermore the Sudan chick produces defective feathers. Unfortunately, Riddle does not give data concerning the body growth, and thus the extent of the injurious action of Sudan III in his experiments cannot be judged. As has been stated already, the majority of investigators fed Sudan III to the adult animals. This may account for the fact that they did not notice any toxic action of the Sudan. It is also possible, however, that rats are more susceptible to Sudan III than either rabbits or guinea-pigs. This point needs to be further investigated.

Since the test rats in the earlier series received Sudan III mixed with olive oil, while the control rats did not get any oil

at all in their ration, it is conceivable that the olive oil might be the cause of this phenomenon, and not the Sudan dye itself. In fact, it has been noted by Adler ('11) that olive oil is highly toxic to rabbits and when given to the young, the growth is
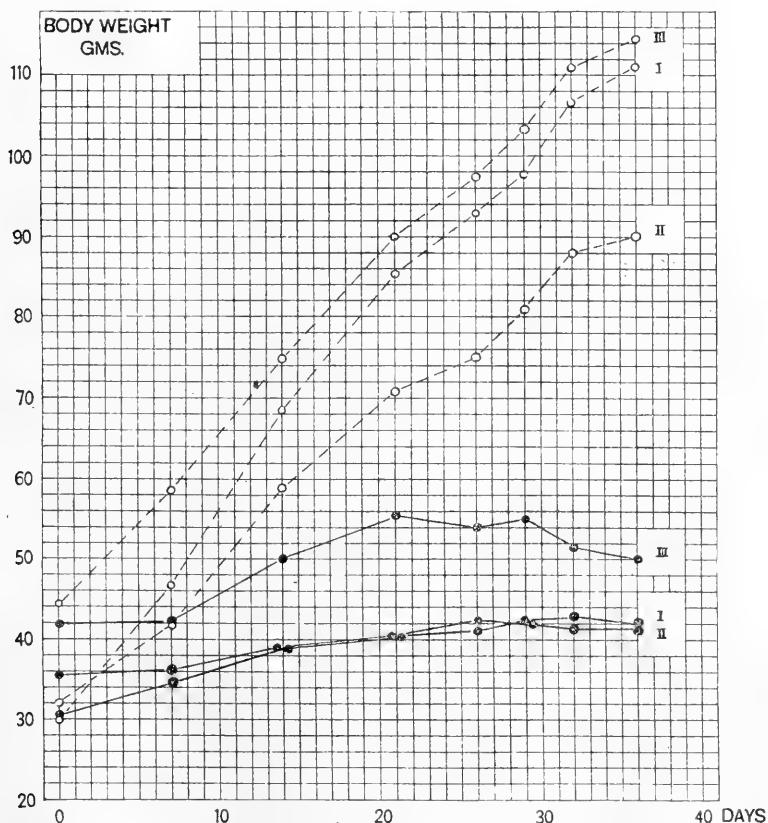
Chart 1.   Showing the effect of feeding a small quantity of Sudan III on the growth of the young albino rats.   Series I, II and III.
○ – – – ○ Control   ●————————● Experimented

materially retarded.   He states that 6 cc. per kilo killed in six days when fed daily; 5 cc. per kilo weight did not kill, but produced secondary anemia with blood crises presenting the picture of pernicious anemia.

I have therefore carried two series of experiments in the following manner. One half of the litter received biscuit and olive oil, while the other half received Sudan III in addition. The amount of oil given was the same in both series. The results show, table 1, that the olive oil alone does not modify the growth of the body and, furthermore, the organs in the controls were not at all modified (table 4). The growth of the rats fed with
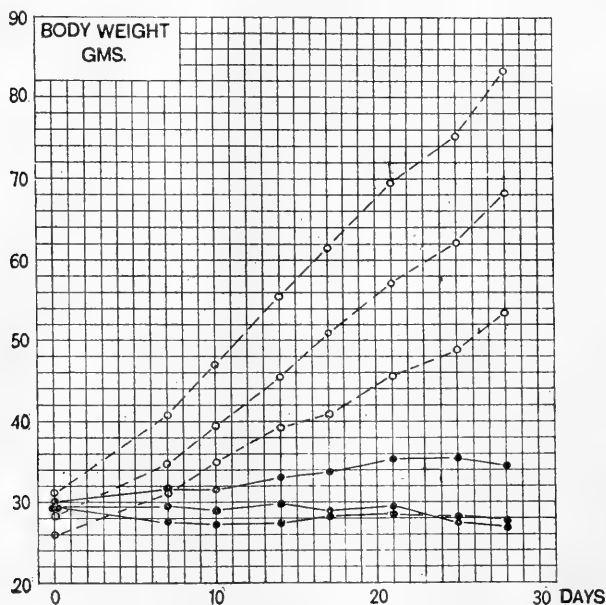
Chart 2. Showing the effect of feeding a small quantity of Sudan III on the growth of the young albino rats. Series VIII.
o – – – – o Control   ●————● Experimented

biscuit plus oil and the rats fed with biscuit plus oil and Sudan III is shown in chart 3. I am therefore confident that the olive oil alone, as here used, is not toxic to the growing rat when given to the amount of 1 to 2 cc. per rat per day. It must be stated, however, that I have not yet tested the rat with higher doses of olive oil.

GENERAL APPEARANCE OF THE RATS FED WITH SUDAN III

The rats fed with Sudan III appear like those severely under-fed. The hair becomes rough and erected, the spine is curved, thus producing an appearance of elongated limbs. Neverthe-less, the rats are very lively and hop around and play with each other as actively as the controls. The urine becomes deep pink usually within two or three days after the Sudan III feeding is
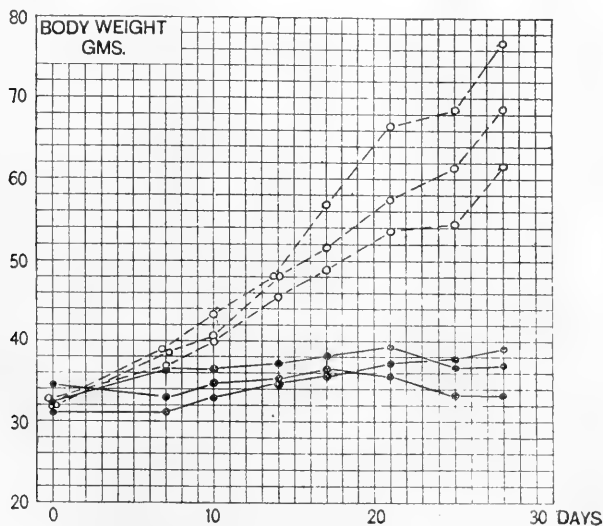
Chart III. Showing the growth of the albino rats which received a small quantity of the olive oil in the normal ration contrasted with the rats which received in addition a small quantity of Sudan III.

o – – – – o   Control (oil fed)   •————•   Experimented

begun. The feces are hard and black in color and ether extrac-tion reveals the presence of the dye in a considerable amount in them. The rats are eager for the experimental ration for the first two or three days, but afterwards they consume but very small quantities. The skin around the urethral opening is usu-ally wet and stained pink, owing possibly to lipuria. I have not determined the maximum length of time during which the rat can survive on this Sudan III ration.

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TABLE 2

Showing the growth of the organs of the albino rat as affected by feeding with Sudan III plus oil. Dose 10 to 15 mgms. Younger series

LENGTH = MM. WEIGHT = GRAMS	INITIAL MEASURE- MENT	SUDAN III AND OLIVE OIL FEEDING			CONTROLS
	$3 \, \male + 5 \, \female$	5 days $1 \, \male + 3 \, \female$	7 days $3 \, \male + 1 \, \female$	10 days $3 \, \male + 1 \, \female$	$1 \, \male + 2 \, \female$
Body length..................	94.8	94.7	94.5	96.3	117.3
Tail length....................	78.4	78.5	79.3	79.8	96.0
Body weight..................	22.9	21.5	23.1	24.3	44.2
Brain........................	1.369	1.332	1.383	1.331	1.463
Kidneys.....................	0.319	0.292	0.323	0.354	0.566
Liver........................	1.260	1.557	1.987	2.324	3.102
Spleen.......................	0.066	0.051	0.065	0.065	0.218
Pancreas.....................	0.166	0.200	0.226	0.243	0.449
Thymus......................	0.043	0.012	0.012	0.007	0.127
Suprarenals..................	0.009	0.009	0.010	0.010	0.014
Thyroid......................	0.005	0.005	0.005	0.006	0.008
Hypophysis..................	0.0014	0.0012	0.0013	0.0014	0.0020
Testes.......................	0.130	0.098	0.087	0.087	0.157
Ovaries......................	0.0086	0.0051		0.0038	0.0084
Age (days)...................	28	33	35	38	38

GROWTH OF ORGANS AS AFFECTED BY THE FEEDING OF SUDAN III

In order to determine the effect of Sudan III on the growth of the organs, young rats just weaned were treated in the following manner.   One or two rats were measured and the weights of the organs determined at the beginning of the experiment.   The remaining rats were examined at different intervals in order to compare the organ weights at later ages with those at the beginning of the experiment.   At the end of the experiment the rats which had been kept as controls and which were receiving the normal ration were examined together with the last test rats.

The object of this final procedure was to show the amount of growth the organs should have made had the rats not been subjected to the Sudan III diet.   The last test group was also utilized to determine the percentage of water and the lipoids in the organs.   The results of these examinations are given in table 2 for the younger, and table 3 for the older series.

TABLE 3

Showing the growth of the organs of the albino rat as affected by the feeding of Sudan III. Dose 8 to 9 mgms. Older series

LENGTH = MM. WEIGHT = GRAMS	INITIAL MEASURE- MENT	SUDAN III AND OLIVE OIL FEEDING			CONTROL
	3 ♂ + 6 ♀	7 days 5 ♀	14 days 5 ♂	35 days 3 ♂ + 3 ♀	2 ♂ + 3 ♀
Body length...................	108.1	113.6	116.3	119.5	155.2
Tail length...................	86.2	94.8	99.0	109.0	139.4
Body weight..................	33.9	37.1	40.1	44.7	98.4
Brain........................	1.379	1.412	1.412	1.438	1.598
Kidneys......................	0.461	0.473	0.482	0.446	0.794
Liver........................	2.063	2.577	2.887	2.615	3.861
Spleen.......................	0.142	0.151	0.222	0.273	0.550
Pancreas.....................	0.280	0.302	0.341	0.421	0.703
Thymus......................	0.107	0.073	0.067	0.031	0.273
Suprarenals..................	0.014	0.013	0.014	0.013	0.023
Thyroid......................	0.008	0.014	0.015	0.013	0.020
Hypophysis..................	0.0016	0.0018	0.0019	0.0019	0.0040
Testes.......................	0.202		0.199	0.0229	1.262
Ovaries......................	0.0107	0.0084		0.0065	0.0152
Age (days)..................	29	36	43	64	64

From table 2 we note that despite the fact of almost no growth of the entire body of the test animals, such organs as the liver and pancreas made a striking increase in weight throughout the entire period of the Sudan III feeding. On the other hand, the thymus gland was greatly atrophied, and the sex glands also showed a marked diminution in weight. None of the other organs showed noticeable loss when compared with those of the rats examined at the beginning of the experiment.

These alterations just noted appear also in the second series of the experiments (table 3), where a much smaller dose of Sudan III (8–9 milligrams) has been given. I may add here that although on the average the kidney weights of the Sudan-fed rats do not differ much from their initial weights, nevertheless the individual records show that in the majority of cases the kidneys of the test rats have suffered a slight atrophy. I am thus inclined to believe that the kidneys also undergo a slight atrophy as the

TABLE 4

The weights of the organs determined in both control and Sudan III fed rats at the end of the experiment

LENGTH = MM. WEIGHT = GRAMS.	SERIES IV		SERIES VII AND VIII		SERIES IX AND X	
	Control 1 ♂ + 1 ♀	Dose 8–9 mgms. Sudan and oil fed for 21 days 2 ♂ + 1 ♀	Control 2 ♀	Dose 10–15 mgms. Sudan and oil fed for 28 days 2 ♀	Control oil and biscuit 2 ♂	Dose 8–9 mgms, Sudan and oil fed for 28 days 2 ♂
Body length.........	129.0	102.7	146.5	110.0	142.5	109.5
Tail length..........	113.0	83.7	137.0	105.0	130.0	101.0
Body weight.........	52.0	26.7	79.8	36.8	71.9	33.9
Brain...............	1.490	1.338	1.516	1.433	1.604	1.478
Heart...............			0.372	0.190	0.371	0.191
Kidneys.............	0.473	0.274	0.721	0.394	0.641	0.423
Liver...............	2.219	1.992	3.073	2.046	2.588	2.174
Lungs...............			0.516	0.344	0.488	0.277
Spleen..............	0.255	0.123	0.167	0.079	0.148	0.074
Pancreas............	0.425	0.268	0.686	0.365	0.591	0.348
Thymus..............	0.146	0.015	0.256	0.030	0.206	0.012
Suprarenals.........	0.015	0.009	0.025	0.010	0.019	0.011
Thyroid.............	0.009	0.010	0.020	0.010	0.013	0.008
Hypophysis..........	0.0023	0.0013	0.0029	0.0018	0.0029	0.0019
Testes..............	0.341	0.071			0.818	0.201
Ovaries.............	0.0097	0.0070	0.0149	0.0052		
Age (days)..........	48	48	50	58	57	57

result of feeding Sudan III. Some of the organs, however, increase in weight. The increase of the liver in weight seems to be due to an accumulation of fat, as this can be shown histologically, as well as by the amount of lipoids extracted (page 113). The increased weight shown by the pancreas may be due to the hyperactivity of this organ in connection with the metabolism of fat.

The atrophy of the thymus gland as the result of feeding Sudan III is most noticeable. The rate of thymus atrophy seems to depend on the amount of Sudan III administered. A larger dose appears to reduce the thymus much more quickly than a smaller dose. Since the thymus is highly sensitive to almost any abnormal physiological condition, such as an exposure to Roentgen rays, inanition, etc., we may assume that the present

TABLE 5

Showing the percentage of water in the various organs of Sudan III fed rats contrasted with those of control rats

	SERIES IV		SERIES III, V AND VI		SERIES VII AND VIII		SERIES IX AND X	
	Control 1♂+1♀	Sudan III 2♂+1♀	Control 1♂+2♀	Sudan III 2♂+1♀	Control 2♀	Sudan III 2♀	Control oil and biscuit 2♂	Sudan III 2♂
Body length......	129.0	102.7	153.0	120.2	146.5	110.0	142.5	109.5
Body weight......	52.0	26.7	99.0	46.4	79.8	36.8	71.9	33.9
Age, days.........	48	48	63	63	58	58	57	57
Blood.............	81.6	83.0	80.7	83.0	80.9	80.8	81.6	82.1
Brain.............	79.5	79.0	79.0	78.6	79.0	78.6	79.1	78.6
Heart.............			77.5	77.6	77.0	76.8	77.3	77.2
Kidneys..........	76.8	76.0	75.8	75.4	76.3	75.3	76.0	76.7
Liver.............	73.1	67.1	70.9	68.2	71.3	67.2	71.7	72.7
Spleen...........	77.5	77.5	77.2	76.7	78.1	76.5	77.5	76.7
Lungs............			78.2	80.2	79.2	80.0	79.4	79.8
Pancreas.........	72.4	76.9	67.9	75.1	69.9	74.6	69.2	76.0
Thymus.......,...			78.6	80.9	79.7	80.5	79.7	78.1

instance of atrophy may be due either to the abnormal nutritional condition or possibly to a direct toxic action of Sudan III on the thymus.

I have given in table 4 (see also the last two columns in tables 2 and 3) the weights of the organs determined in both control and test rats at the end of the several experiments.

The organs given in table 4 were used for the determination of water and lipoid content, as well as for the demonstration of the presence or absence of Sudan III in various organs, by both microscopic and extraction methods. As we should expect, the organs of the test rats are smaller than those of the controls. It is interesting to note also that even the liver and pancreas, although they have made a continuous growth during the experimental period, are yet far behind those of the control rats. I wish to call special attention to the case of the control rats which received the same amount of olive oil as the test rats (series IX and X). In this case we notice that the control and test rats show the same degree of difference in the organ weights as in

TABLE 6

The percentage of water in the brain of Sudan III fed rats contrasted with that in the brain of the control rats

AGE	NO. SEX	BRAIN WEIGHT, GRAMS. PERCENTAGE OF WATER		AGE	NO. SEX	BRAIN WEIGHT, GRAMS PERCENTAGE OF WATER	
		Control	Sudan III			Control	Sudan III
days				*days*			
35	C = 1♂ + 2♀	1.407	1.331	57	C = 1♂	1.492	1.422
	S* = 2♂ + 1♀	80.070	79.820		S = 1♂	79.160	78.700
35	C = 1♂ + 1♀	1.414	1.324	57	C = 1♂	1.712	1.535
	S = 1♂ + 1♀	80.560	79.960		S = 1♂	79.000	78.500
36	C = 2♂ + 1♀	1.440	1.377	60	C = 1♀	1.598	1.470
	S = 2♂ + 1♀	80.240	80.070		S = 1♀	78.730	78.330
37	C = 1♀	1.483	1.415	61	C = 1♀	1.561	1.528
	S = 1♂	79.920	79.720		S = 1♂	78.910	78.620
48	C = 1♂ + 1♀	1.490	1.338	61	C = 1♀	1.750	1.534
	S = 2♂ + 1♀	79.450	79.030		S = 1♀	79.060	78.930
55	C = 1♀	1.434	1.397	68	C = 1♂	1.596	1.455
	S = 1♀	79.170	78.780		S = 1♂ + 1♀	79.000	78.370

*S. Sudan III fed.

the other cases where their controls did not receive any oil at all. This shows that the olive oil alone, in the amounts here given, does not produce any noticeable effects on the organ weights. The percentages of water in the various organs are given in table 5.

From table 5 we see a considerable difference in the water content between the control and test rats. The organs which give a diminished percentage of water are the liver, spleen, kidneys, and brain, while those which give an increased percentage are the blood, pancreas, and lungs. The greatest difference between controls and test rats is shown in the blood, pancreas, and liver. Although the brain gave a very small difference in the water content, nevertheless this difference is highly constant. With a view to illustrating the constancy of difference in the percentage of water in the brain, I have compiled table 6.

In this table the means from all the twelve series of experiments are given. We notice that in every instance the percentage of water in the Sudan III brain is less, and thus the difference in the water content between the control and the test rats cannot be doubted.

The full meaning of these alterations in the percentage of water in the several organs I am unable to explain; nevertheless, it seems evident that in the case of the liver the diminution of water is mainly due to the accumulation of fat. From two determinations I have obtained the following results for the amount of fat in the liver:

	CONTROL	SUDAN III	
	per cent	*per cent*	
Case 1.....................	19.02	35.52.	Cold ether only used.
Case 2.....................	20.94	36.95	Ether and alcohol extraction.

The histological examination shows clearly that the greater part of the liver was infiltrated by fat.

In the case of the brain I found a significant increase in the amount of lipoid in the test rat, as will be seen from the following:

	CONTROL	SUDAN III
	per cent	*per cent*
Alcohol-ether extract.....................	42.15	43.94

This increase in lipoid may account for a slight diminution of water content in the test rat, but this requires further study.

A slight diminution of the water content in the case of the kidneys and the spleen may also be due to a slight increase of l pin content as in the case of the brain, but I have no data to prove this. The increased water content noted in the lungs and pancreas I am also unable to explain at this moment. It seems, however, highly probable that in the case of the pancreas, hyperfunction in connection with the metabolism of fat may account for this.

As to the high water content of the blood of the rats fed with Sudan III we may attribute it to the condition of profound anaemia. The blood examination kindly made by Dr. Rivas (University of Pennsylvania) shows a loss of 12 to 40 per cent in the number of erythrocytes. I have made an alcohol-ether extraction of the dry blood and obtained the following results:

	CONTROL	SUDAN III
	per cent	per cent
Alcohol-ether extract.....................	1.56	3.66

This higher content of fat in the blood may indicate that the elimination of fat from the blood has been much disturbed.

GENERAL REMARKS

Various alterations produced as the result of feeding Sudan III to the growing albino rat have been presented. We found that the alteration is not limited to a mere retardation of the growth of the body and organs, but that the composition of these organs is also modified. It is strange that most previous investigators who fed Sudan III to rats, as well as to several other animals, failed to notice any toxic effect of this dyestuff. This failure was perhaps due to the fact that most investigators used adult animals for their investigation, without very careful testing, while Sudan III is strongly toxic only to the growing animals.

Riddle, who fed Sudan III to young chicks, noticed a retardation of the growth of the body, as well as defective feathers. This investigation by Riddle, on one hand, and my own, on the other, shows clearly that Sudan III is injurious to young animals at least. I have already mentioned the high resistance of the rats whose bodies weigh more than 50 grams, but I have not tested the reaction of the fully grown adult rats to Sudan III. I have, however, tried feeding Sudan III to female rats which were nursing young. In these cases a toxic action of Sudan III was found without exception. The lactating glands react promptly to the dye, and failure of the secretion of milk is evident within a day

or two. Thus in many instances I was obliged to give the mother food free from Sudan III in order to keep alive the young rats which were depending on her. So far as these nursing female rats were concerned, the effect of Sudan III is similar to that in young rats which have just been weaned; that is, it arrests growth and causes emaciation, curvature of the spine, rough hair, etc. No examination of the organs in the suckling young has yet been made. It seems clear from this that Sudan III is toxic to nursing females, as well as to their suckling young.

It is of interest to note that extraction with ether shows a small trace of Sudan III in the following organs: liver, pancreas, lungs, and kidneys, but fails to show even a trace of Sudan III in the brain, spleen, or heart. Corper ('12) demonstrated the presence of Sudan III in the liver, and often in the lungs, of guinea-pigs, but always failed to find it in the brain, spleen, heart, testes, or adrenals. Mendel and Daniels ('12, '13) found Sudan III in the liver (four out of five cases), but never in the kidneys of the rat. This discrepancy as to the presence of Sudan III in the kidneys might be due to the fact that the rats used by the present writer were young and were also heavily dosed.

Highly interesting are the questions how Sudan III produces such profound alterations in the growth of the body and organs and how we are to explain the chemical alterations found in the organs.

Riddle, who noticed a retardation of growth and the formation of defective feathers in the chicks fed with Sudan III, considers that the stained fat becomes less easily available to the organism and thus Sudan III produces effects similar to simple starvation. This question of the availability of stained fat was taken up by Mendel and Daniels, who, however, combat Riddle's conclusions and think that the stained fat is just as readily available as unstained. Although I have not made a special study on this point, nevertheless it seems clear that the alterations found in the test rats cannot be explained, as merely the result of simple inanition, as proposed by Riddle. A high degree of anaemia, fatty infiltration of the liver, as well as the nephritic condition, point to pathological alterations rather than to the phenomenon of simple inanition.

It appears to me, on the other hand, that in the test rats the normal metabolism of fat must have been much disturbed, as can be seen from the presence of a large quantity of fat both in the liver and blood, and thus we may consider that the stained fat also is less readily utilized.

Another interesting question is the origin of the lipoids contained in the organs. We found that Sudan III is practically absent, even after five weeks of continuous feeding, in nearly all the organs so far examined. Particular interest attaches to its absence from the brain in which nearly 50 per cent of the solids are represented by lipoids. Moreover, the amount of Sudan III to be recovered from the liver, pancreas, lungs, and kidneys is almost negligible. Microscopical examination fails completely to reveal any trace of Sudan III or stained fat in the organs even when the extraction mass yields a trace of it. Since Sudan III is so intimately combined in fat, and wherever fat appears Sudan III is always found, and furthermore since Sudan III stains not only fat or oils, but fatty acids also, it seems probable that if the cell elements should absorb the fat or fatty acids for building up the organ lipoids, we might expect Sudan III to be absorbed also. There is no a priori reason to suppose that Sudan III alone is rejected, while the fat or fatty acids containing it are capable of being absorbed.

This result suggests that the lipoids found in the organs may not be formed by the fat or fatty acids furnished from without, but are elaborated by the organ itself; in other words, the organ lipoids are endogenous and not exogenous in origin.

CONCLUSIONS

1. Albino rats 27 to 33 days old were fed with Sudan III mixed in olive oil. The amount of Sudan III given daily was about 8 or 9 milligrams in one series and 10 to 15 milligrams in the other. A dose of 20 milligrams was found to be too large.

2. In all cases Sudan III retards to a considerable extent the normal rate of the body growth. Olive oil alone as given by me does not produce this effect.

3. Most organs maintain nearly their original weights; some change in weight. The liver and pancreas show a steady increase, while the thymus, testes, and ovaries show a striking diminution. The rapidity of the thymus atrophy seems to be proportional to the amount of Sudan III administered.

4. The rats fed with Sudan III show a high degree of anaemia, and the reduction of erythrocytes may be as high as 40 per cent.

5. The composition of the organs is more or less altered. We find an increase of water content in the blood, lungs, and pancreas, while a reduction occurs in the liver, spleen, kidneys, brain, and heart.

6. The reduction in the water content of the brain belonging to the Sudan III fed rats is small, but it is highly uniform and occurs in all of the series without a single exception. It was noted also that the brain belonging to the Sudan III fed rats gives an alcohol-ether extract nearly 1.5 per cent greater than in the control brain.

7. The alcohol-ether extract of the dry total blood is significantly greater (2.1 per cent) in the Sudan III fed rats than in the controls.

8. Extraction with ether shows a small trace of Sudan III in the following organs: liver, pancreas, lungs, and kidneys, but failed to show even a trace in the brain, spleen, or heart.

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FURTHER STUDIES ON THE MODIFICATION OF THE GERM-CELLS IN MAMMALS: THE EFFECT OF ALCOHOL ON TREATED GUINEA-PIGS AND THEIR DESCENDANTS

CHARLES R. STOCKARD AND GEORGE N. PAPANICOLAOU

From the Department of Anatomy, Cornell Medical School, New York City

NINE FIGURES

CONTENTS

1. INTRODUCTION

The present contribution presents the results obtained during
the sixth and seventh years of an experiment on the modification
of mammalian germ cells by the treatment of parental generations
with alcohol. A number of new facts are added to our previous
findings, and the data now permit a more thorough analysis.
Treating the results obtained during these two years separately
may be looked upon as taking a cross-section of the entire experi-
ment. And when this isolated portion of the investigation is
compared with the previous studies, it supplies a further most
important control for the experiment as a whole.

The earlier reports of this investigation (Stockard, '12, '13,
and '14; Stockard and Papanicolaou, '16) were made after the
first two years, three years, and five years of its progress. These
reports showed, in what seems to us a definite way, that the germ
cells in either the male or female mammal may be changed or
affected by a chemical treatment administered to the body of the
individual. The progeny derived from such chemically treated
animals showed more or less marked deviations from the normal
in many definitely measurable qualities, such as their mortality
records, structural appearance, nervous reactions, and ability
to reproduce. The treatment also affected in the male, the crucial
germ-cell test for mammals, their ability to beget offspring when
mated with normal females.

In general it may be stated that the offspring produced when
treated males were paired with normal females were inferior in
several respects as compared with other offspring from the same
normal mothers bred to control males of exactly the same original
stock. Further, when the male offspring from treated fathers

were mated with normal females, the individuals resulting from such matings were as a group decidedly inferior to the young produced by normal females when mated with control males. This group inferiority was not only present in the grandchildren, or F_2 generation, but also in the F_3 generation descended from alcoholized great-grandparents.

Fortunately, since these experiments were first reported, several similar studies by other investigators using the methods here employed have been conducted on other mammals and birds. Our results have been corroborated, though the response to the treatment has in some instances been thought to differ from that shown by the guinea-pigs. Useful and suggestive interpretations of the results have been advanced, yet certain points of view are presented with which we are not always able to completely agree. The bearing of these studies on the present results will be discussed in a section beyond.

In particular we are indebted to Pearl ('17) for his recent characteristically clear and exact analysis of the influence of alcohol fumes on domestic fowls and their progeny. This study has suggested to us the importance of a considerable amount of data contained in the card-catalogue records of our animals which had not been fully valued in the previous discussions of the experiments. In the present report we have followed several of Pearl's ideas in more completely separating the qualities to be contrasted between the alcoholic and control lines.

As might be expected, various objections have been advanced from time to time regarding the cause and explanations of the results which we have reported on the effects of alcohol in the guinea-pig. In all cases, however, the objections have been raised either by persons entirely unfamiliar with these animals and their breeding qualities or by others who have not been sufficiently interested or careful to read the descriptions of the animals and breeding methods used. It has been suggested on certain occasions that the defects and degenerate conditions which have been reported in our alcoholic lines were probably present in the original stock on which the experiment was conducted. Such a remark in the face of the experimental control

which has been fully described scarcely warrants discussion, yet we should like to state in the beginning for the benefit of the casual critic who may not wander through the following pages the real nature of the original control.

A group of forty animals, eleven males and twenty-nine females, was obtained from a reliable breeder in the early fal of 1910. These animals were all under one year old and strong and vigorous in appearance; most of the females were pregnant. All the females were kept until they had produced a normal litter of young. Their production was what would ordinarily be obtained from healthy guinea-pigs; all of the young were normal in appearance and about 80 per cent of them survived under the by no means perfect system of care then employed.

Three males and six females, after the test matings, were then taken for alcohol treatment. The choice was entirely random, there being no evident marks of superiority or inferiority in any of them as compared with the other animals retained as normal control. One of the three males selected for treatment lived to be more than seven years old, and the others were all healthy, strong animals that lived long and bred vigorously. These treated males were mated with alcoholic females and with normal females. The same normal females were mated at different times with normal males and such offspring were considered control. From the beginning of the experiments it may be said that the same normal female often serves as part of the experiment, being mated to alcoholic males and again as the control. The same is true of normal males; they are frequently mated successively with alcoholic females and normal females. From this original stock the normal animals, both males and females, have invariably given rise to average normal offspring when paired with normal mates, while, on the other hand, the treated animals being part of the same breed, have in the quality of their offspring shown a decidedly inferior condition even when paired with normal mates.

After the experiment had been in progress for eighteen months, in March, 1912, a new stock of animals of an entirely different source from the first lot was introduced. Again, after testing

their breeding ability by one normal mating, certain of this lot were taken for alcohol treatment, and these animals were bred both separately and with the original lot.´ Yet the records of the alcoholic and normal individuals were again different

Finally, in October, 1915, when the experiment was five years old, we obtained four new stocks of guinea-pigs from different dealers and introduced them into the experiments in various ways along with our now pedigreed lines from the old stock. The records of these new animals as well as our old lines known for three or more generations regarding inbreeding and other conditions are to be considered in the present paper. These experiments bring out additional facts in the study, and we believe they supply an unquestionable control on the previous results. In other words, this may be taken as a new study considering the conditions of 1,170 guinea-pigs born from various alcoholic lines as well as from normal control animals. About 600 of the animals are born of alcoholic lines with no inbreeding in any case back through their great-grandparents. About 300 of them are from alcoholic lines and at the same time somewhat inbred; these are for all considerations treated separately from the straight alcoholic lines. The control animals with which the alcoholics are compared are of the same blood lines as the alcoholics and are also not inbred.

2. QUALITY OF THE EXPERIMENTED AND CONTROL ANIMALS

a. *Selection of animals*

As briefly mentioned above, the control and the first treated individuals are derived from exactly the same original stock. During the progress of the experiment other animals have been subjected to the treatment, and these in many cases are of known pedigree for several generations in our colony. In all cases only vigorous animals are used for the treatment and they are invariably tested by being mated at least once before the treatment is commenced. This precaution is undoubtedly of much importance, equally as important as knowing the blood lines, in selecting normal breeders. These test matings are

further strengthened by the fact that the same normal males are mated with alcoholic females and with normal females, and normal females are mated with alcoholic males and again with normal males as a control, etc. In this way the experimental and control animals are actually in some cases the same individuals and in all cases they are constantly being bred together. There is no question that the animals treated with alcohol and the control are equally general or random samples of the population. Yet there is a marked contrast between the records of their offspring and descendants.

b. Inbreeding

The alcoholic lines which we shall analyze in detail in the following considerations are practically devoid of inbreeding. Almost all of these animals are known in our colony for three or more parental generations, and we mean in stating that they are not inbred that a given individual in their ancestry never appears more than once back through the great-grandparent generation. In the first table to be considered the straight alcoholic lines may be compared with other lines that are not only alcoholic, but also inbred, usually to a slight degree, and it is seen that inbreeding in either the alcoholic or the control to a limited degree gives no indication of any significantly injurious effects.

In our former report ('16) there were shown to be more injurious effects in the alcoholic inbred lines than in the noninbred. This difference has now disappeared on account of the fact that the animals in the former table were more closely inbred and were earlier generations than the bulk of those in the present consideration. The degree of inbreeding in the inbred lines is now much reduced as compared with the earlier table, and the records have improved. This difference between the earlier and the present results indicates that inbreeding in these alcoholic lines may be easily carried to a degree which will make the injurious effects more marked. We have avoided even the slightest approach to such a degree of inbreeding in the straight

alcoholic lines. The pedigrees of a great majority of these alcoholic animals could readily be given to cover several generations, but it does not seem advisable to enter into this detail, since there is no possible chance that any differences which might exist between the normal and alcoholic lines are due to different degrees of inbreeding among the individuals of the two groups. And further, in all the groups it is entirely out of the question that any difference between the records of the control and the records of the alcoholic may be due to the control having been by chance originally good breeders and the alcoholics originally bad. The control animals are in almost all cases either sisters, brothers, parents or other blood relations of the treated animals.

c. The number of animals alcoholized

Recognizing the great variability in the breeding results from the different individuals in a group of higher animals, such as mammals, it has been deemed entirely essential to make our experiment on a considerable number of males and females. The mating records of two normal male guinea-pigs are frequently quite different even though paired with the same females. It is also highly probable that different individuals will differ in their susceptibilities and responses to the treatment, so that the records of two or three males might easily prove confusing even though all might exhibit some effects of the experimental treatment. Thus the following twenty-eight males have been treated with alcohol, and a number of matings from each of these and their descendants have supplied the breeding records. The first three are from the original 1910 stock. Nos. 4 ♂, 5 ♂, and 6 ♂, and the remaining twenty-five are animals bred and reared in the colony or from the newly introduced stocks: Nos. 43 ♂, 45 ♂, 70 ♂, 72 ♂, 80 ♂, 81 ♂, 678 ♂, 887 ♂, 913 ♂, $\frac{AN}{A}$ 129 ♂, 157 ♂, 168 ♂, 183 ♂, 302 ♂, 353 ♂, 365 ♂, 574 ♂ $\frac{AA}{A}$ 493 ♂, $\frac{(NAp)(AA)}{A}$ 771 ♂, $\frac{\frac{AN}{A}N}{A}$ 889 ♂, 1091 ♂, 1134 ♂, 1153 ♂, 1326 ♂ and 1327 ♂.

In the case of the females an attempt has also been made to lessen the error caused by indiv dual differences in breeding capacity and in responses to the treatment by using a number of animals. Thirty-four individuals have been treated in all. Many of these females were bred for a number of times as control before being subjected to the fume treatment, after wh ch they are placed of course among the alcoholics. Their earlier breeding records are therefore part of the control data and their subsequent records part of the data included for the alcoho ic lines. The same thing is true of a number of the males mentioned above. In none of these cases can it be objected that the animals had become too old for normal vigorous breeding while being used in the alcoholic lines. We have constantly guar ed against breeding the alcoholic animals after there is any question as to age affecting their breeding capacities when compared w th the normal breeding cycle of these guinea-pigs. The treatment of the large majority of the animals is begun when they are less than one year old, and they have a vigorous breeding span of at least four years. The individua¹ females wh ch have been subjected to the alcohol fumes are the following: The first six are from the original 1910 stock, Nos. 8 ♀, 9 ♀, 10 ♀, 11 ♀, 12 ♀, and 34 ♀; the following twenty-eight are animals reared in the colony or from the newly introduced stocks: Nos. 55 ♀, 57 ♀. 59 ♀, 60 ♀, 61 ♀, 62 ♀ 64 ♀, 65 ♀ 66 ♀, 88 ♀ 90 ♀, 117 ♀, 158 ♀, 161 ♀, 654 ♀, 847 ♀, 865 ♀, 946 ♀, $\dfrac{NA}{A}$ 122 ♀, 200 ♀

228 ♀, 397 ♀, 1139 ♀, $N\dfrac{\frac{NA}{A}}{A}$ 796 ♀, 1002 ♀, 1105 ♀, $N\dfrac{\frac{NA}{A}}{\frac{A}{A}}$

1468 ♀ and 1469 ♀.

There are no contrasts between the histories and capacities of the experimented and control animals that can be fairly accounted for as due to differences in either their origins, blood lines, or relationships. As far as experiment and control with biological material may be practically useful, any differences which may exist between the records of the alcoholic guinea-

pigs and the normal control lines are due to the treatment administered to the alcoholic lines. We further believe that if the differences which do exist between the alcoholics and control are so slight that the crudest mathematical calculations are insufficient to indicate their presence, the experiment has then produced no data of biological interest or importance since conducted on animal material of such complexity as a group of mammals. This statement is made with no intention or presumption to question the real importance and value of modern biometrical methods, but is only what we believe should apply to this particular experiment.

3. EXPERIMENTAL METHOD AND THE CARE OF ANIMALS

Throughout these experiments alcohol has been administered to the guinea-pigs by a method of inhalation which was devised in the beginning. The animals to be treated are placed in fume tanks fully described and illustrated in an earlier communication (Stockard, '12) and absorbent cotton soaked with commercial 95 per cent ethyl alcohol is placed on the floor of the tank beneath a wire screen on which the animals stand. The fumes of evaporating alcohol very soon saturate the atmosphere of the tanks and the guinea-pigs introduced into this saturated atmosphere are allowed to remain until they show distinct signs of intoxication. During the earlier years of the experiment they remained for one hour each day in such tanks, but during the past twelve months we have increased the treatment to two hours per day for the males and three hours for the females.

This longer treatment is much better in that the animal, of course, gets a larger dose and its tissues may become more quickly influenced by the treatment. The animals may remain until they are completely intoxicated, in which case they are unable to walk, and therefore lie in a typical drunken stupor, or they may be affected to such an extent that they attempt to walk and in so doing stagger and fall in a manner characteristic of the drunken state. The amount of treatment here employed, however, does not produce complete intoxication.

It would be perfectly possible with an elaborate system of measurements to determine exactly the quantity of alcohol fumes

each individual receives per day, per month, or per year, but, as we have pointed out before, such knowledge would be of no advantage either to us or to others in estimating the results of these experiments. No two individuals would be affected to exactly the same degree by the same dose, and as is the case with man the later influences of the treatment no doubt differ in different individuals. There is also no particular interest here in the amount of alcohol used, since our primary problem is whether or not an active chemical substance may be given in sufficient amounts to the parent mammal to produce effects upon its offspring or descendants by modifying its germ-cells, or in the case of the pregnant female by acting through the mother on the developing embryo.

We have thus employed, as stated in our previous reports, a simple physiological index of the amount of treatment, giving enough each day to perceptibly influence or intoxicate the animals, but not enough to produce a complete drunken stupor.

Animals may remain for very long times in these treatment tanks when alcohol fumes are not present without in any way suffering for want of breathing space. This method has many advantages so far as the general health of the individual animal is concerned over drinking alcohol into the stomach, as will be discussed in the following section.

The only object in choosing alcohol as the treating agent is on account of the fact that considerable knowledge exists as to its physiological actions on certain animal tissues and it is known to be an active organic substance that might produce effects. It had further been used by one of us (Stockard, '10) in producing various developmental abnormalities in fish embryos which could be treated directly with diluted alcohol, and the general nature of the effects on these embryos had been studied. A final advantage in using alcohol in such experiments is the ease with which it may be administered to the animals by the inhalation method which we have described.

Caging and care of animals. All of the guinea-pigs, both the experimental and the control animals, are kept in the same type wooden cages. These are group cages, each containing twenty

compartments one foot high by one foot wide by two feet long. Each compartment is sufficient to fully accommodate one female with her litter of young or three adult animals. In all of the cages some of the compartments are occupied by the alcoholic animals and others by the control so that the cage accommodations for the two classes are identical. The cages are thoroughly cleaned, the floor sprinkled with sawdust and fresh hay put in daily. In addition to the hay, which is eaten with relish, the animals are fed every day with fresh carrots and several times per week oats are given with occasional cabbage or kale. It is also important for their perfect health, though not necessary for their existence, that guinea-pigs be given fresh water every day during the warmer months and several times per week during the winter. This is frequently neglected in keeping these animals since it is commonly thought that they get a sufficient amount of water from the green foods. At the present stage of this experiment, along with several other problems now being studied, a stock of over 500 animals is constantly kept on hand. One reliable keeper devotes his entire time to cleaning the cages and feeding. He in no case discriminates in his treatment of different animals and from the cage numbers is unable to know all of the alcoholic line animals or the controls.

From the beginning of this experiment, in making the matings a male is placed in a compartment with one female during her heat period (Stockard and Papanicolaou, '17); in this way there is no opportunity for preferential or choice matings. A male might discriminate in his behavior between an alcoholic and a normal female if in a compartment with the two, as Pearl believes his roosters have done when placed in a pen with both normal and alcoholic hens. After the male has remained in the pen for one month, the female is carefully examined and at this time with some practice the investigator may feel the small embryos in the horns of the uterus. The male is removed and the female remains alone in the compartment. A list of all pregnant animals, both alcoholic and control, is kept and their compartments are examined both morning and late afternoon of each day in order to detect an abortion should it occur, since the

female may devour the early aborted young. In addition to this, each pregnant female is reëxamined once or twice and the number of fetuses in the right and left horns of the uterus recorded each time on her catalogue record.

By this method it has been found that a number of females may often absorb their embryos, either one or all, and so give birth to a smaller litter than originally began development or to none at all. The absorption of individual embryos seems so far .as we have detected not to interfere with the development of the remaining ones. These examinations of pregnant females have been repeatedly controlled by opening the animals and observing the contents of the uterus, and the examinations in all cases have been very accurate. This thorough watch over the females has furnished us much more exact data as to prenatal deaths, early absorptions, etc., than were contained in our former reports.

The entire care of the animals has been much improved during the past two years. Our records for monsters and other weakened conditions are, therefore, somewhat reduced; yet the same marked contrast between alcoholic and control is present even though the weakened alcoholic lines have no doubt profited more by the improved methods of care and feeding than have the healthier controls. The defects are also the same in type as those formerly observed, though not so marked in degree.

4. THE INFLUENCE OF ALCOHOL INHALATION ON THE INDIVIDUAL

The immediate effects on guinea-pigs of inhaling alcohol are somewhat similar to those observed after drinking it. As stated above, the animals after some time become unable to walk without staggering as a result of loss of muscular coördination and finally reach, with a long treatment, a state of complete alcoholic stupor.

The presence of alcohol in the blood of the guinea-pig after the inhalation treatment is readily detected by even simple chemical tests, as we have frequently pointed out. Other investigators also find that alcohol is easily introduced into the

general system of birds and several mammals by this method.
Pearl ('16 b) definitely recognizes the fact that alcohol is readily
taken into the system by the inhalation method, but makes the
following statement regarding the effect: "It is true that it is prac-
tically impossible to induce by the inhalation method in animals
habituated to alcohol that state of muscular incoördination which
is usually, but by no means always, the most striking objective
symptom of the condition of being drunk." Our observations
on guinea-pigs show them to respond very differently in this re-
spect from the fowls used by Pearl. In the case of guinea-pigs
habituated to alcohol, it is very easy by the inhalation method
to induce a state of muscular incoördination due to the drunken
state and finally a complete anaesthesia, the muscles being en-
tirely relaxed and the animal unable to move. It may be that
fowls are peculiar in their reaction to alcohol and it may also be
extremely difficult to administer to them a highly effective dose
without fatal results. Such an idea is suggested by the fact that
Pearl does not get the gross symptoms of intoxication by leav-
ing his fowls in the tanks for one hour, yet they "accumulate a
fatally toxic dose of alcohol by staying in the same tank under
the same conditions for from twenty minutes to half an hour
longer." Guinea-pigs do not at all react in this manner after
an hour or two in the tank they may show signs of intoxication
by becoming groggy, with their muscles generally relaxed so that
when lifted their bodies are almost entirely limp. Yet they
have not consumed anything near the fatal dose, since they may
remain in the same tank under the same conditions for even two
or three hours longer before becoming completely intoxicated
so as to be unable to move; and in order to inhale a fatal dose
they must remain still longer, at least six or seven hours

We have treated only one fowl, a white leghorn cock, in our
tanks. This bird responded much as the guinea-pigs do show-
ing decided muscular incoördination, staggering and frequently
almost falling as it walked. He was also able to withstand a
long treatment and never, though treated several times, did he
show any tendency to suddenly accumulate a fatally toxic dose
as Pearl found his fowls to do.

a. Contrast between the immediate effects of alcohol taken by inhalation and by stomach

An important point to keep in mind when considering these animals intoxicated by the inhalation method is that on being removed from the tanks they use up the alcohol in their systems very rapidly and also begin to throw off alcohol by respiration. The intoxication is, therefore, of short duration so that the animal may be fairly well recovered within half an hour or perhaps only a few minutes, depending upon the amount of the treatment. In other words, this is an acute short intoxication closely comparable to an ether anaesthesia from which the animal readily recovers when the fumes are no longer inhaled, but which during the inhalation may give a complete intoxication. On the other hand, a drunken condition resulting from taking alcohol into the stomach is of much greater duration since the gradual absorption of the alcohol continues for a longer time before the system begins to burn it up or throw it off to such a degree that the amount present begins to be continuously reduced, permitting the animal to slowly recover from the drunken state. A guinea-pig receiving a dose of about 25 cc. of 15 per cent alcohol into its stomach will be decidedly intoxicated within fifteen or twenty minutes, and the extent of intoxication will increase until the animal becomes unable to walk or stand and lies in a drunken stupor. Such a condition may persist for six or seven hours or longer, and the body temperature may be lowered from one to even four degrees Fahrenheit.

It seems to us, therefore, that the chief difference between inhaling alcohol and drinking it into the stomach is that in the first case the action of the substance on the animal system is of shorter duration, lasting but little longer than the length of the sojourn in the fume tanks—a short acute effect—while alcohol in the stomach is gradually and continuously absorbed for a considerable length of time so that the animal's tissues are acted upon for hours after receiving the dose. Another very serious phase of the stomach alcohol, aside from the typical intoxication effects, is its tendency to derange the animal's powers of diges-

tion and thus to cause very injurious results. The inhalation method is accompanied by no such complications.

We have now considerable data bearing on this problem and are conducting an experiment to determine the quality of the effects on the animal body and the progeny produced when dilute alcohol is taken into the stomach of guinea-pigs for long periods of time. The results of this study are to be compared with the data from the fume-treated animals.

b. The vigorous condition of the animal after daily inhalation of alcohol for long periods

A number of the guinea-pigs have now been treated with alcohol fumes almost to a state of intoxication six days per week for from five to six years. Few guinea-pigs in captivity live so long a time. There were two males treated for over six years, one of which lived to be more than seven years old. So far as we know, this is the longest life reported for a guinea-pig. The treatment was continued with these very old animals but they were not used for breeding. In no case when the treatment was begun on an animal over three months old could any injurious effects on its general welfare or length of life be discovered. We have called attention to these facts in our previous publications.

There are certain direct injuries resulting from the inhalation of ethyl-alcohol fumes during the early stages of the treatment. The mucosa of the respiratory tract is considerably irritated during the first few months and secretes freely while the animals are in the tanks, causing a watery flow from the nostrils and mouth. The membranes become more resistant as the treatment goes on and later little effect can be noticed. This irritation has never given rise to any noticeable inconvenience to the animals. The surface of the eye is also greatly irritated during the first few months, causing an abundant secretion from the lachrymal glands while in the fume tanks, and finally resulting in many instances in an opacity of the cornea. In some cases this opacity disappears after a few weeks and the animal is again able to see, yet some of the animals treated for several years have remained entirely blind,

A number of the treated animals have died and many others have been killed at various times during the progress of the experiment. Their organs and tissues have been carefully examined at autopsy and later studied microscopically. All tissues have appeared practically normal and none of the various well-recognized pathological conditions occurring in human alcoholism have been discovered. Tissues from animals treated as long as three years have been carefully studied, and the heart, stomach, liver, lungs, kidney, and other organs present no noticeable conditions that might not be found in normal individuals. Alcoholized animals are usually fat, but no fatty accumulation has been noted in the parenchyma of any organ.

Several males and females have been semicastrated during the experiment, and the ovaries and testes have been found to be in a generally healthy condition. It has seemed, however, that the ovaries of treated animals as well as all animals of the alcoholic lines show an unusual tendency to become cystic as compared with the ovaries of normal individuals. We have not, however, made sufficient comparisons to give the foregoing statement any greater weight than a mere supposition.

The general condition of all animals under the fume treatment is particularly good, and, as stated above, they continue to grow if the treatment is begun on individuals before they have attained full size, and all become fat and vigorous, taking plenty of food, living long, and behaving in a typically normal way.

The accompanying illustrations of five treated animals photographed along with control individuals show their perfectly normal appearance. In figure 1 is seen two male guinea-pigs and from the photograph as well as in life it would be impossible for any one to detect signs of physical inferiority on the part of one or the other. Yet the animal on the right, No. 80 ♂, was four days less than 5 years old when the photograph was made and had inhaled alcohol over one hour per day, a sufficient dose to give signs of intoxication, for six days per week, during four years, two months and five days. During the last seven months he had inhaled alcohol fumes two hours per day. He is perfectly well and alert, as the photograph clearly shows. His

companion on the left is a normal animal, No. 150 ♂, being 4 years and 3 months old when photographed. The sober existence of this male has not given him any advantage in appearance over the old alcoholic; both are very good males, each weighing almost 900 grams when photographed. This is well above the weight of the ordinary adult guinea-pig.

Figures 2 and 3 show again on the left the same normal animal, No. 150 ♂, in order that the reader may obtain a more definite impression of the uniformly good condition of the three alcoholic males. The alcoholic male No. 72 on the right in figure 2 was 5 years, 1 month and 10 days old when photographed,
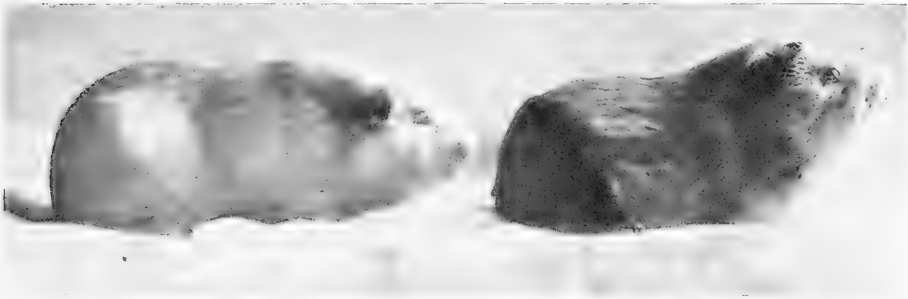
Fig. 1   The animal on the left is a normal male, No. 150, over four years old. The one on the right, No. 80 ♂, is almost five years old and had been treated with the fumes of alcohol six times per week for four years and two months, yet is seen to be in a vigorous condition.

weighing over 900 grams. He had been treated with alcohol fumes one hour per day until the last seven months, when he was treated two hours per day for six days per week. The entire duration of his treatment when photographed was four years, two months and five days.

Figure 3 shows on the right alcoholic male No. 70. This animal was 5 years, 1 month and 11 days old when photographed, and weighed 885 grams when 5 years old. He had received the same amount of alcohol treatment as the other two. The three were bred and reared in our colony and are above the average male guinea-pig in size and vigor. They have been good breeders as young normal specimens, as well as during

their alcoholic careers, but there has been a decided difference in the quality of their offspring during the two periods.

Figures 4 and 5 show two female alcoholics photographed along with the same black male No. 116. In figure 4 the alcoholic is an albino, No. 65 ♀. She was introduced into the experiment with the second stock from a new source in March, 1912. This female had been treated with alcohol fumes for two years, seven months and seventeen days when photographed. During the first two years of the treatment she inhaled one hour per day for six days per week and during the remaining seven months was treated for three hours per day, until fairly well intoxicated each time. The normal male No. 116 was 4 years, 8½

Fig. 2   The animal on the left is the same control individual, No. 150 ♂. The one on the right is an alcoholic male, No. 72, which was more than five years old and had been treated with alcohol fumes for four years and two months.

months old when photographed. The female No. 65 gave ₁ ormal young before her treatment began, but now produces offspring with very poor records.

The female No. 158 is shown on the left in figure 5. This animal was produced in our colony from normal parentage and was 4 years and 3 months old when photographed. She had been treated for fourteen months one hour per day and for three hours per day during the last seven months. She is a large vigorous female. These photographs illustrate to some extent the fact that the treated animals themselves are little changed or injured so far as their normal appearance goes, and should there be inferior qualities in their offspring these cannot

be attributed to a condition of general depression in the parents, but more clearly to a peculiar action of the strange chemica material in the blood upon the glands of reproduction or the germ cells of the males and females.

In his study of the influence of alcohol inhalation on the domestic fowl, Pearl has found the treated individuals to respond in a way closely similar to our treated guinea-pigs. He has fortunately reported his results in much more thorough detail than we, yet the facts contained are practically the same for the two groups of animals. The mortality records of treated fowls show an advantage over similar records from untreated
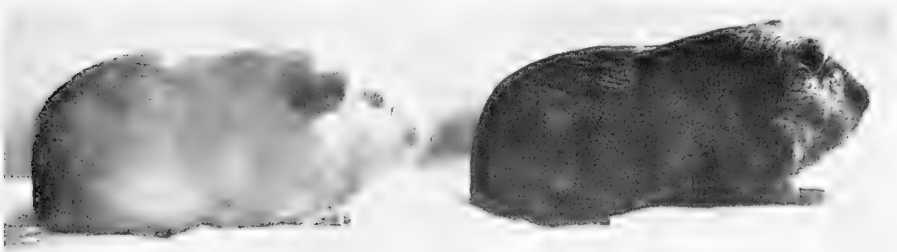
Fig. 3 Two male guinea-pigs. One on the left the normal animal, No. 150, more than four years old. On the right, No. 70 ♂, more than five years old and had been treated with alcohol fumes for four years and two months.

control. Our card catalogue contains the record of every death that has occurred among the guinea-pigs since the beginning of the experiments, and we may state in a general way that the mortality statistics for the treated animals is certainly as good and perhaps slightly better than those of the control.

Pearl has very naturally considered these findings in connection with the "widespread popular opinion that life-insurance statistics have 'proved' that even the most moderate use of alcohol definitely and measurably shortens human life." On careful investigation of the statistics Pearl finds them to be entirely unconvincing and to be based on biological evidence insufficient to prove anything. This is exactly in line with our own experi-

ence in studying the literature of any phase of human alcohol-
ism. We have studied very thoroughly the literature relating
to the influence of alcoholism in men and women on their prog-
eny and, including the study of Elderton and Pearson, find it
to suffer from the defects which Pearl points out in the longevity
studies. Some of these contributions we shall discuss beyond,
but none give any exact statement as to the amount of alcohol
consumed or the length of time during which it had been con-
sumed or any definite information as to other conditions or the
general behavior of the individuals considered. The data are
usually collected by persons entirely untrained and incapable of

Fig. 4 On the left normal male No. 116, almost five years old, and on the right an alcoholic female, No. 65, more than five years old that had been treated with alcohol fumes for about two and one-half years.

accumulating biological evidence. These extremely inexact
records are often subjected to very careful and exact mathe-
matical analysis which tends to give a scientific aspect to the
consideration, but in no way improves the quality of the incor-
rect data used. Unfortunately, this renders it difficult to make
comparisons between the responses of human alcoholics and
those of selected animals used in well-regulated experiments.

Yet aside from the above, even should the data relating to the
influence of alcohol on human longevity justify a comparison
with experimental results, we feel that such a comparison could
not properly be made with either Pearl's observations on the
effect of alcohol on the mortality record of fowls or ours on the
life record of alcoholized guinea-pigs, since in both experiments

the animals have been treated by inhalation of alcohol fumes, while human alcoholics have taken the substance into the stomach.

The difference between the effects on the treated individual of the two methods of administering alcohol cannot be too strongly urged.   By the inhalation method the individual experiences only the stimulating or with further dosage the intoxicating and anaesthetizing effects of alcohol.   As far as we have detected there are no injurious secondary effects on the individual's welfare resulting from habitual inhalation of ethyl-alcohol fumes.   The results are very different, however, when the guinea-pigs drink daily doses of 15 per cent ethyl alcohol.
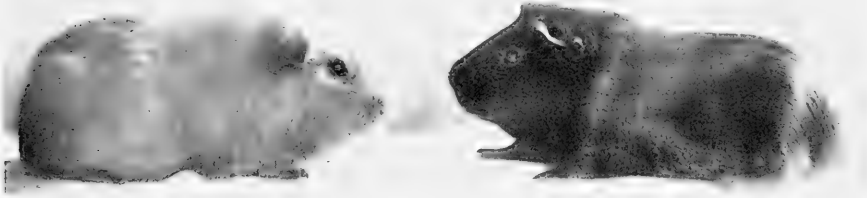
Fig. 5   The same male, No. 116, is shown on the right and an alcoholic female, No. 158, is on the left.   She was more than four years old and had been treated with alcohol for over two and one-half years, yet she is in no way injured in appearance.

Only a few animals and a short time are sufficient to demonstrate the fact.   A number of animals were given alcohol into the stomach at the beginning of these experiments and their digestion and metabolism were so deranged by the treatment that we were forced to devise and adopt the inhalation method as a more likely means of conducting an experiment of long duration.

It has been shown by us for guinea-pigs, and Pearl has demonstrated with fowls, the prosperous manner in which animals withstand the inhalation of alcohol vapor.

We may now give briefly the effects on three guinea-pigs of drinking daily doses of alcohol for only three weeks.   The animals, Nos. 173 ♀, 1098 ♂, and 1184 ♂, at the beginning of the

experiment weighed respectively, 822, 635, and 527 grams, the female being old and the two males young growing specimens.

They were each given about 20 cc. of 15 per cent ethyl alcohol in tap water daily, except that once each week they were given almost 30 cc., which was a completely intoxicating dose. The 20-cc. dose causes all of them to be groggy for a few hours after drinking it; the effect increases for an hour or so and then gradually wears off. There is only slight if any change in rectal temperature. The animals seem fully recovered on the following day and have a normal appetite, but do not eat so ravenously as do untreated individuals.

When 30 cc. of 15 per cent alcohol is given in three 10-cc. doses at fifteen-minute intervals the animal is badly intoxicated and unable to walk within fifteen or twenty minutes after the last dose. The hind legs are particularly uncertain, the animal often tumbling over almost on its back, kicking frantically and having great difficulty in righting itself. Should its mouth come in contact with food the guinea-pig will chew in a peculiar manner, seeming in all reactions to be typically drunk. After one and a half or two hours the animal lies on its side with its trunk muscles often undergoing spasmodic contractions several times per minute, if taken up or made to move it struggles and falls panting in the drunken condition. By this time the body temperature may have fallen as much as 2 degrees below the pretreatment record. After three hours it is still unable to stand or walk and is breathing heavily with a temperature as much as $2\frac{1}{2}$ degrees Fahrenheit below normal. After four hours the condition is about the same and so for several hours longer until it gradually begins to recover and by the following morning it is fully recovered, but shows in its appearance the effects of the experience of the previous day.

When animals are given five partial and one complete intoxication by stomach alcohol per week they begin after a few days to regurgitate some of the stomach contents on receiving the first swallow or so of alcohol, but after this they take the dose without further disturbance, though they resist taking it more and more each time. Their desire for food is somewhat reduced as the treatment is continued.

After the first week No. 173, the old female that should have weighed the same or gained in weight under normal conditions, had lost 50 grams, or 6 per cent of her total weight.   The two young males should have gained, No. 1098 gained 17 grams, only 2.6 per cent of his weight, while No. 1184 lost 4 grams or practically stood still.   Their weight records for the indicated intervals are as follows:

	173 ♀	1098 ♂	1184 ♂
	grams	grams	grams
May 7........................	822	635	527
May 14........................	772	652	523
May 21........................	740	656	477
May 28........................	759	659	469
June 2........................	735	637	483
June 11........................	775	621	475

The alcohol was taken from May 7 to 28, and during that time the first guinea-pig lost 63 grams, or 7.6 per cent of its original weight.   Of the two young males one gained 24 grams, or 3.7 per cent of his weight, while the other lost 58 grams, or 11 per cent of his original weight.   During the next two weeks after the treatment stopped the male that had gained 24 grams lost 38 grams, so that at this time each animal weighed less than when it began to take alcohol.

This may have been a rather strong dose, but allowing for that, it was readily recognized that these animals were suffering from the treatment, while other guinea-pigs inhaling alcohol for three hours per day until groggy showed no injured appearance.   Animals taking alcohol into the stomach suffer mainly on account of the injurious effects on their digestion.   Alcohol acts on the gastric mucosa in such a way that the individual is placed at a disadvantage in handling its food and the ill effects observed are more largely due to this derangement of digestion than to the toxic action of alcohol on the animal system.   Alcohol in the stomach makes the case complex, while we believe that inhaling alcohol gives effects simply due to the chemical action of alcohol itself on the tissues.   For these reasons we do not believe that comparisons are easily made between the conditions of animals

that have inhaled alcohol fumes and the conditions of other animals that have taken alcohol into the stomach, since the latter individuals may be reacting more to a deranged digestion than to alcoholic intoxication. Therefore, there is objection to making comparisons between the mortality records of animals treated with alcohol by the inhalation method and the reports on the effect of alcoholism in man.

Yet, on the other hand, it may be possible that the influence of alcohol on the germ cells of an animal is the same whether the alcohol reaches the reproductive glands by being inhaled into the lungs or swallowed into the stomach. Such a position is not inconsistent with the discussion above if we take into account the possible, though unknown, effects of the deranged metabolism of the parent on the germ cells.

5. A GENERAL COMPARISON OF THE PROGENY FROM ALCOHOLIC LINES WITH THOSE FROM NORMAL LINES

The consideration above has brought out the fact that the inhalation of alcohol fumes sufficient to produce partial intoxication six times per week for long periods does not cause any easily recognized disadvantages in the general bodily condition or powers of existence of guinea-pigs. Pearl's experiments demonstrate the same fact in connection with the domestic fowl. This is, o course, leaving out of account the irritating effects of the fumes on the surface of the eye which may result in bl ndness, although even this is no handicap to either feeding or eproduction under cage conditions. I, then, the general body tissues are not sufficiently injured to cause an easily noticeable change in their powers of function, why should the germ cells be particularly susceptible to the treatment? The germ cells within the body of a mammal are undifferentiated generalized cells with no known function except to exist and await their time to develop. The soma or body, in respect to the germ cells, is simply a culture medium in which they live. The nourishment necessary for their existence is delivered to them by the body fluids. Any strange chemical substance which may find its way into the body fluids will reach the germ cells, and should this substance be sufficiently active and injurious in its effects

the germ cells may be so modified as to render them incapable of normal development.   This might easily occur without differentiated somatic tissues being sufficiently damaged to greatly impair their usual functions.   In other cases, and probably as a rule, the somatic tissues are also injured by any offensive substance present in sufficient quantities to modify the germ cells, and there are many reasons for believing this to be the result in several chronic human infections.

One must not infer from these statements that the germ cells are readily injured by poisons taken into the system; indeed, they seem on the contrary to be protected to a remarkable degree against such effects, and for this reason it is difficult to obtain a substance which may be used in experimental studies on the modification of the germ cells.

Should the germ cells be modified through the action of any substance, the point of particular importance is that all cells arising from such a modified germ will be similarly modified, since they are merely products of its division, and thus the soma and germ cells of the resulting individual will deviate from the normal in proportion to the degree of the primary modification of the cells from which it arose.   Provided the change is one of such a nature that the cell or its parts are unable to recuperate, for example, if their specific chemical or physical make up be altered, then not only will the generation resulting from the originally modified germ cells be affected, but all future generations arising from this modified germ plasm will likewise be affected.

It seems also highly probable that should such results occur, the modifications to be observed in the somatic generations will be of a generalized nature affecting the organism in various ways so as to render its development less vigorous, its chance of survival less certain, and its ability to behave in a normal fashion more or less hampered.   In certain cases the animal might really show no evident signs of its altered character.   It seems to us, on the other hand, that only through the very rarest chance, one in possibly thousands, would any of the small number of definite characters under observation happen to be modified by their response to the treatment.   The inheritance of coat-color,

for instance, may not be affected, although the germ plasm might be so seriously altered as to give rise to the most extremely abnormal individuals. The same would apply to the very few other characters in mammals the inheritance of which have been studied from the Mendelian standpoint.

Finally, then, the fact that the soma seems little injured by the alcoholic inhalations is in no way an index of what may be expected from the development of the germ cells of guinea-pigs which have been under habitual treatment.

Arlitt and Wells have very recently reported that the administration of alcohol in the food of male white rats for two or more months results almost constantly in the appearance of marked degenerative alterations in the testicles although other organs were apparently uninjured. They find that these changes affect the steps of spermatogenesis in inverse order to their occurrence, so that for some time before sterility and complete aspermia result, the animal is producing spermatozoa with all possible degrees of abnormality. The probable relation of such phenomena to the production of defective offspring is obvious.

A general survey of the progeny from the normal and alcoholic lines as a whole will first be undertaken and is based on the data presented in table 1. In this table the animals are arranged in four groups, the first column containing the records of those produced by normal control matings without inbreeding, the third column records of normal animals somewhat inbred, while the second column gives similar records for animals produced in the alcoholic lines without inbreeding, and the fourth-column animals are not only alcoholic, but also somewhat inbred. The table contains in all records of 1170 animals, from our catalogue numbers 613 to 1909 except 126 animals that could not properly be included such as 39 new stock adults, 22 killed for different purposes during early embryonic life, 31 derived from mothers with only one ovary, and others too heterogeneous in origin, as those from ancestors treated during pregnancy, etc., to be certainly placed. They represent, as stated above, the animals produced during the sixth and seventh years of the experiment and none from the earlier years.

The figures of the first horizontal space may be used to indicate

TABLE I

MORTALITY AND QUALITY RECORDS OF THE PROGENY FROM NORMAL AND ALCOHOLIC LINES

	Normal lines					Alcoholic lines					Normal inbred					Alcoholic inbred				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Total number	10	46	90	72	15	38	162	279	100	15	0	8	21	12	0	13	80	144	40	25
	24.03%			37.33%	233	33.61%			19.32%	594	19.15%			29.26%	41	50.19%			21.52%	302
	Average litter 2.77					Average litter 2.47					Average litter 2.02					Average litter 2.60				
	Mat. fail. 4 (4.54%)					Mat. fail. 36 (13.04%)					Mat. fail. (2.5%)					Mat. fail. 16 (13.12%)				
Lived over 3 months	10	39	75	45	12	31	132	170	48	2	0	8	16	8	0	13	53	98	13	7
	100%	84.78%	83.33%	62.5%	80%	81.51%	81.47%	60.93%	48%	13.55%		100%	76.20%	66.6%		100%	66.24%	53.05%	32.50%	28%
Absorbed, Premature, Stillborn	0	4	7	16	0	4	19	79	34	12	0	0	2	4	0	0	19	27	15	16
	(51.92%)					(70.14%)					(66.66%)					(65.25%)				
Died within 3 months	0	3	8	11	3	3	11	30	18	1	0	0	3	0	0	0	8	19	12	2
	(48.08%)					(29.85%)					(33.33%)					(34.74%)				
Total dead	0	7	15	27	3	7	30	109	52	13	0	0	5	4	0	0	27	46	27	18
		15.21%	16.66%	37.5%	20%	18.42%	18.51%	39.06%	51%	86.66%			23.80%	33.33%			33.13%	31.94%	61.5%	72%
	(22.31%) = 100					(35.52%) N 18.79% = 189					(21.95%) N 22.46% = 98					(33.07%) N 18.59% = 210				
Defective	0	0	0	0	0	0	2	12	2	0	0	0	0	0	0	0	8	8	1	0
							0.61%	4.30%	2.0%								1.25%	5.55%	25%	
						2.52%										(3.31%)				
Oversized (more than 500 gr. when 3 months old)	3	2	5	0	0	3	2	3	0	0	0	0	0	0	0	0	3	3	0	0
	36%	10.86%	5.55%			7.89%	6.9%	1.07%								13.58%	3.73%	2.08%		
	(5.58%)					(2.86%)										(2.64%)				
Undersized (less than 300 gr. when 3 months old)	0	2	0	0	0	0	2	2	4	0	0	0	0	0	0	1	3	3	4	2
		2.17%				1.23%	0.11%	4.0%								1.63%	3.73%	1.38%	7.5%	8.0%
	(0.42%)					(1.34%)										(3.64%)				

the productivity of the different lines. The numbers 1 to 5 indicate the number of young, one, two, three, four, or five produced by a female in a single litter. Litters of five individuals are the largest that have occurred from this strain of guinea-pigs. The average number of young in a litter from the normal lines is 2.77, and of the 233 animals included in this column 24.03 per cent of them were born in litters of one or two young. About 39 per cent were born in litters of three, while 37.33 per cent of the animals were members of large litters of four and five individuals. There were only a few normal inbred animals, as shown in the third column, but their general occurrence in the different-size litters was about as in the straight normal lines, half of the animals were born in litters of three, and almost 30 per cent in larger litters, and only about 20 per cent in litters smaller than three. The average litter happens to be in the small number of inbred animals a little higher than in non-inbred stock.

The arrangement of the young in large and small litters in the alcoholic and alcoholic inbred lines is almost exactly the reverse of what we have just seen for the normal. Again, a little less than half of the animals occur in litters of three. But over 30 per cent of the individuals are from litters of only one or two, while about 20 per cent are born in litters of four or five. Stated in other words, in the normal lines one and one-half times as many individuals are born in litters of four or five as in litters of one or two, while in the alcoholic lines one and one-half times as many are born in litters of one or two as in litters of four or five.

The explanation of this, we believe, is as follows: About half of the pregnancies in this stock of guinea-pigs should result in litters of three, as is found to be the case in all of the lines of table 1. All litters of less than three young are due in the first place to a low productivity on the part of the female as is probably indicated by the production of more than one-fifth of the normal young in such litters. In the second place, small litters are frequently due, particularly in the alcoholic lines, to the death and absorption in utero or early abortion of one or more members of an originally large litter. The absorption in utero of such embryos, often of rather large size, may occur in a nor-

mal guinea-pig, yet such a phenomenon is not very common, although in the alcoholic lines it is frequently observed. We shall consider this process below, the only point of interest here being its effect on the size of the litter.

The exactly reversed percentages of individuals born in large and small litters in the normal and alcoholic lines, as shown by the table, may indicate that one-third of the animals in alcoholic lines that are born in litters of one or two were originally in litters of three, four or five. For example, the normal lines have in all well over 12 per cent more animals born in litters of four or five than in litters of one or two, and the alcoholic lines have over 12 per cent more in litters of one or two than in litters of four or five, and this 12 per cent probably has been thrown from the larger into the smaller litters on account of early abortions and absorptions which occur in the former. The too frequent occurrence of small litters is undoubtedly indicative of not alone an actually low productivity, but a very early prenatal mortality.

Another occurrence also partly due to an early prenatal mortality is the failure of a mating to produce a result. No doubt in rare cases fertile guinea-pigs may be mated during the heat period of the female, as these have been, without a following conception. In the normal lines four out of eighty-eight matings, or 4.54 per cent, failed, giving negative results, while in the alcoholic lines three times as many matings failed, and very probably this excess represents those cases in which not only a part of the litter is lost through an early prenatal mortality, but the entire litter is destroyed. Of course, some cases of actually infertile matings are also represented.

By this 'early prenatal mortality' is meant the absorption or loss of an embryo before it is of sufficient size to be detected on carefully feeling the uterus through the body wall of the mother. With experience an embryo eight or ten days old may be detected by an external examination of the uterus. Through our routine examination of the females after being with the males for one month, any embryo lost after this time will have been discovered and is definitely recorded in the third horizontal space of the table. If absorption or early abortion of one or more embryos in a litter may actually be observed to occur after as

much as ten days of development, there must certainly be a pre-
natal mortality of some extent previous to this time. Experi-
ments with the eggs of lower forms which develop outside of the
mother, permitting direct observation, speak for the great pre-
ponderance of an early embryonic mortality, many such eggs
dying during the cleavage and gastrular stages when subjected
to even slightly unfavorable conditions. We have some direct
evidence on 'early prenatal mortality' in female guinea-pigs
which have been examined by operation after repeated 'mating
failures.' The ovaries of some such animals contain corpora
lutea of pregnancy indicating that an embryo had been present
shortly before the examination.

Pearl records that the eggs from alcoholized fowls are to a
high degree infertile. This he believes is due to many of the
germ cells as such having been killed by the treatment. By
infertile, Pearl means, of course, that no fertilization or zygote
formation took place, yet it is extremely difficult in all cases to
detect whether the early stages of development may not have
occurred and been followed by death and degeneration. The
death may have occurred during the cleavage or gastrular stages
while the egg was yet in the uterus of the hen and many of
the 'infertile eggs' might really be classed among the early pre-
natal mortalities. We make these suggestions merely as pos-
sibilities which to us are somewhat tempting, since if there was
actually an early prenatal mortality in some of these 'infertile
eggs' it would bring the effects of the alcohol treatment on the
fowls and mammals still closer together. It is only through our
recent analysis of the size of litters and mating failures, along
with careful examination of the pregnant females, that we have
become aware of the sometimes frequent very early embryonic
death.

The second horizontal space shows the number of young from
the several lines that reached maturity, or lived over three
months. Here again the size of the litter is an important factor.
It may be stated generally that the power of survival of a guinea-
pig varies inversely with the size of the litter in which it is
born. We shall see beyond that this is also true of their birth
weight, growth rate, and certain other qualities so that in mak-

ing comparisons between young guinea-pigs it is important to know whether the individuals concerned occurred in litters of equal size.

In the normal lines all individuals born singly survived, and, as the seventh space shows, 30 per cent of them were unusually large or over size when three months old. Normal animals born in litters of two or three survive in about 84 per cent of the cases and are often of large size. The members of litters of four survive in only 62.5 per cent of the cases and are not generally vigorous animals. The records show that 80 per cent of the young in litters of five survived, but this is very unusual and is due probably to the small number involved, and possibly to a slight extent to the extreme care with which the pregnant females with the larger number of young were handled. This extreme care, however, only saved 13.33 per cent from the same number of alcoholic-line young born five in a litter.

The second column indicates that over 81 per cent of alcoholic animals born in litters of one or two are capable of survival. Such a record is almost as good as the control, showing how very strong the members of small litters are and indicates again that an early individual selection may have played some part, since no doubt there has been a prenatal mortality among the weaker individuals which originally existed in some of these litters. This is emphasized further by the fact that the members of litters of three survive in only 60.93 per cent of the cases. Here the prenatal mortality has not played so severe a rôle and many weaker individuals are born. The power of survival of animals born three in a litter from the control is about 23 per cent better than from the alcoholic lines. Only 48 per cent of the alcoholic-line individuals from litters of four were able to live three months. Recognizing the small numbers involved, only 13.33 per cent of the alcoholic guinea-pigs born in litters of five were viable. It thus appears that when the alcoholic animals produce large litters the quality of the young is very poor, whereas their small litters contain animals with good survival records. There is little doubt that this apparent difference in quality is in part due to a prenatal selection which, in the case of the small litters, has eliminated most of the weaker individuals and left only the

stronger to be born. In addition to this, it must also be recognized that the ability of the female to properly nourish the members of the large litters is somewhat overtaxed. Three or less than three embryos are very well nourished by normal mothers. It must be recognized here that the inferior records of the alcoholic lines are not alone produced by alcoholic mothers, but come also from alcoholic fathers as following tables will show.

The survival records of the normal inbred lines are about the same as those from the straight control, and are almost equally superior to the alcoholic lines.

The alcoholic inbred animals have a survival record closely similar to the straight alcoholic lines, and again decidedly inferior to either the normal or normal inbred lines.

The fifth horizontal space contains the mortality records which are the reverse of the survival records just considered. However, we have given here not only the actual mortality in litters of different sizes, but have corrected the total mortality record on the basis of the occurrence of large and small litters and their mortality in the different lines as compared with the control. We have also expressed the mortality in numerical proportion in the several lines, taking the control as 100. The total mortality in the normal lines is 22.31 per cent. This is a very good record, since it not only includes the postnatal mortality, but all exact prenatal mortality as well. We mean by exact prenatal mortality those cases of absorption in utero and premature abortion which were actually observed, and not those calculated on the basis of size of litter, mating failures, etc., as was discussed in connection with the productivity of the different lines.

The total mortality of the normal inbred is 21.95 per cent, or almost the same actually as well as when corrected for litter sizes as the straight normal lines.

The total mortality of the alcoholic lines without inbreeding was 35.52 per cent, or almost 1.6 times greater than the mortality of the control. But this does not fully represent the real difference between the two lines unless it be corrected on the basis of the mortality record for the different-size litters in the alcoholic and the normal. The mortality is much higher among

the members of the large-size litters than among those in the small litters, and the large litters are 1.7 times more frequent in the control than in the alcoholic lines.

The mortality is corrected on the basis of the normal records as follows: The rate for the normal animals born one in a litter is zero; two in a litter, 15.21 per cent; three in a litter, 16.66 per cent; four in litter, 37.5 per cent, and five in litter, 20 per cent. On this basis what should be the number of alcoholic animals dying in the several different-size litters? The numbers should be zero instead of 7 for one in litter animals; 24.64 instead of 30 for two in litter animals; 46.48 instead of 109 for three in litter animals; 37.5 instead of 52 for individuals born four in litter, and 3 instead of 13 for five in litter. These numbers give a total of 111.62, which divided by the number of alcoholic animals, 594, shows a mortality percentage of 18.79. On the basis of the control mortality for the different-size litters, this is what the mortality should have been in the alcoholic lines, yet instead of 18.79 per cent it was actually 35.52 per cent, or almost double the normal rate. Again to express the corrected mortality in the alcoholic lines in terms of the control as 100, we find that for every 100 of the control animals that die 189 from the alcoholic lines die.

The last column shows the 302 alcoholic inbred animals to present a still worse record. The actual mortality here is 39.07 per cent, or one and three-fourths times higher than in the control. Here again correcting as in the preceding cases, the mortality on the basis of the control record in the different-size litters, it should normally be 18.59 per cent, but instead the mortality is 2.1 times greater than this among these alcoholic inbred animals. In other words, for every 100 control animals that die 210 alcoholic inbred individuals succumb. While the normal inbred animals, although their numbers are small, present a slightly better record than the straight control, 98 of these dying to 100 of the control.

In the third and fourth horizontal spaces of the table the total mortality is divided into the prenatal and postnatal deaths. The proportion of prenatal to postnatal death in the different lines presents peculiar arrangements that will be seen to exist, not only

in this, but in several of the tables to follow. The prenatal records include embryos that die and are absorbed in utero, never passing to the outside, other embryos and fetuses which die and are passed out or born prematurely, and finally full-term young which die shortly before birth and are, therefore, still born or born dead. The postnatal deaths include all animals dying before reaching three months of age, at which time guinea-pigs are about mature.

In the control lines 51.92 per cent of the total mortality occurred before birth or was prenatal, while 48.08 per cent of the deaths occurred after birth. Considering the numbers involved, it therefore may be said that the pre- and postnatal mortalities are about equal in the straight control lines. There is no evidence here of a particular tendency on the part of the young animals to succumb at any given or critical stage in their development.

The numbers contained in the normal inbred column are certainly too small to be considered.

In both the alcoholic and the alcoholic inbred lines where the numbers involved are considerable (the records showing 329 deaths among 896 animals), the prenatal mortalities are double the postnatal deaths. The alcoholic column shows 70.14 per cent of the total mortality to occur before birth, while only 29.85 per cent of the individuals that died were lost after birth. The last column gives for the alcoholic inbred animals 65.25 per cent of the total mortality as prenatal and only 34.74 per cent as postnatal. This consistent arrangement in the two columns indicates a tendency on the part of the weak and subnormal individuals of the alcoholic lines to succumb during early stages of their development. Such an interpretation is exactly in accord with and is substantiated by the high early prenatal mortality which exists in these lines as indicated by the size of their litters and frequent mating failures when compared with the control.

A mortality arrangement of this kind accords with what is known of almost all weak or diseased stocks—there is a very high loss during the early stages of development, as well as during

later embryonic or uterine life. Furthermore, many individuals die very soon after birth, while those that happen to survive the periods shortly following birth are often capable of an almost or quite normal existence.

The mortality in the control is low, but half of this, or a high proportion, occurs after birth. The mortality in the alcoholic lines is high, but only a low proportion, about one-third of this, occurs after birth. It may be added further that the young alcoholics which die after birth in the majority of cases die within a few days, while the control young that die after birth are more likely to be scattered along over a number of days or weeks.

It is thus seen that in both the alcoholic and alcoholic inbred lines there is a decided tendency for the developing embryos and young to succumb during the early periods of their development. This would suggest that these affected individuals were often incapable of passing through the early critical stages of uterine life. But if they were sufficiently fit to survive these periods, their chance for existence was good, so that their postnatal mortality, although actually higher than the control, was proportionally much lower. Thus we have a somewhat rigid individual selection taking place during the stages of uterine life, so that the sum total of the individuals at a given stage is of a better average quality than during any previous stage and vice versa. Therefore, as is clearly shown beyond, those animals of the alcoholic lines which live to become mature and prove to be fertile are a strictly selected few and in each generation the proportion of strong to weak individuals through this selection constantly tends to increase.

The sixth horizontal space shows a complete absence of defective individuals in either the normal or normal inbred groups. It may be stated here that during the entire seven years of this experiment not one grossly defective or deformed individual has appeared in the nonalcoholic or control lines. This is a rather remarkable record for any group of animals, and it speaks strongly for the perfection of the original stocks from which both the control and the alcoholic lines have been derived.

In the alcoholic lines about $2\frac{1}{2}$ per cent of the individuals were grossly defective. By defective is meant those specimens which show deformities, such as one abnormally small eye, cataract or opaque lenses, deformed limbs, paralysis of the limbs, gross tremors which make the animal incapable of locomotion or proper feeding, etc. There are slightly more defectives in the alcoholic inbred groups, 3.31 per cent in all.

The next line records the over-size or unusually large animals, those weighing more than 500 grams when three months old. Among the control 30 per cent of the individuals born singly or one in a litter grew to be unusually large specimens. More than 10 per cent of those in litters of two were also unusually large, and 5.53 per cent of the three in litter animals are included in this class. None of those born in litters of four or five were able to attain such a size. Of the total control animals over five and one-half per cent were of this large size, while only about half as many from the alcoholic and alcoholic inbred lines attained such a distinction, yet in both treated groups there were over two and one-half per cent of large specimens.

The last line of the table shows the occurrence of unusually small animals, those weighing less than 300 grams when three months old. Among 233 control animals only one such individual appears, 0.42 per cent. The alcoholic lines contain more than three times as many of these as the control, but still very few, only 1.34 per cent. The numbers in the normal inbred column are too small for consideration. Among the 302 alcoholic inbred animals there were eleven under-size specimens, or 3.64 per cent. This is almost three times as high a percentage as occurred in the alcoholic lines and over eight times as high as is recorded for the control animals.

Comparing the present results with those of our earlier papers, particularly with the similar table 2 ('16), it will be noticed that the numbers involved are almost twice as great and the records of the animals considered are decidedly better than were formerly shown. This improvment in the quality of all lines is due to several factors. In the first place, the breeding methods have been decidedly improved since studying the oestrous cycle of

the females and determining the exact time of the 'heat periods' (Stockard and Papanicolaou, '17). This has enabled us to pair the animals at the most favorable periods and thus to obtain far better and more exact mating records than was possible on the basis of the previous conceptions of the guinea-pig's sexual behavior. Secondly, the housing, care, and feeding of the animals are decidedly better during the last three years than during previous times, and on this account the mortality in all lines has been reduced, but as might be expected, the weaker alcoholic lines have profited more by this improved condition than have the control animals. For example, the mortality record of the control has been lowered only a little more than 3 per cent, while in the alcoholic lines it has been lowered a much as 18 per cent. This improvement in the alcoholic lines is also partly due to the existence of more late-generation animals with many normal ancestors. Thus, although the lowered mortality record of the alcoholic may not be entirely due to the better living conditions, yet it serves as a striking illustration of the difference in response to the change on the part of the control animals and the alcoholics. The previous somewhat unfavorable state did not greatly impair the powers of existence of the control animals, but it did evidently eliminate some of the weaker alcoholic individuals that might have survived under more ideal arrangements.

It must be recognized, in the third place, that for the alcoholic inbred animals the degree of inbreeding among the later generations here included is less intense than was the case with earlier generations in the former reports. And for this reason the previous rather decided differences which were shown between the straight alcoholic group and the alcoholic inbred animals have almost, though not entirely disappeared.

Lastly, the fourth point of difference to be borne in mind in comparing the earlier and present records is that there are now more late-generation alcoholic descendants with less affected material in their total germ-cell complex than was true of the animals in the former tables, which as a group were composed of generations closer to the direct alcohol treatment. For ex-

ample, an animal derived from a directly alcoholized father and a normal mother could be said to contain half affected and half normal germ plasm, whereas another in whose pedigree the only alcoholic individual was an alcoholized grandfather, would undoubtedly contain a smaller amount of affected stuff.

Finally, then, in the light of the facts involved, the general table presents an impression closely similar to that derived from the previous records of these experiments, but it adds data of much importance for a clearer understanding of the problems concerned.

The improvement in the present records over the former ones might suggest that should the methods of breeding and caring for the animals reach perfection, the differences between the alcoholic lines and the control might be entirely erased. This would be possible if the improvement was due alone to method, but such a suggestion ignores the fact that the improvement is more largely due to the presence of late-generation animals with only a small amount of alcoholic germ plasm in their ancestry and a large number of normal progenitors. The analysis of the following table 2, in which the several generations are treated separately, will fully substantiate the validity of the foregoing statement.

Before considering this table, however, we may discuss briefly the phenomenon of absorption of embryos in utero and our methods of examining pregnant females in order to fully record the fate of all embryos that begin to develop. A knowledge of this prenatal mortality is involved not only in the table just studied, but in several of those that follow.

6. ABSORPTION OF EMBRYOS IN UTERO AND ABORTIONS OF PARTS OF LETTERS: METHODS OF DETECTING THESE PROCESSES

After having observed the course of pregnancy and the size of the litters produced in a large number of cases, we became convinced that many of the small litters delivered at full term were only partial litters. Particularly in the alcoholic lines it became evident that abortions of one or two members of a litter might

occur without hindering the further development to term of the remaining members. It was also recognized as is known even for the human female that embryos might be absorbed in utero. In the guinea-pig we have found that the absorption of one or more embryos in utero, as is true of partial abortion, may not interfere with the further normal development and birth of the remaining members of such litters.

When it was realized that these absorptions and abortions of parts of litters were taking place, the necessity arose of definitely detecting each case in order to make the prenatal mortality records approach correctness. A systematic examination was, therefore, begun of every female after being with a male for one month up to within a week or ten days of delivery.

The female to be examined is allowed to stand on a flat surface and the investigator with both hands presses the ventral abdominal wall so as to feel with the fingers the horns of the uterus against the dorsal abdominal wall. With considerable practice the small embryos and placentae may be definitely counted within one or both horns of the uterus. The number of embryos and their position in the two horns of the uterus are noted on the record card of the female. After this initial examination she is reexamined once or twice during the pregnancy and each time the number and position of the embryos with the date of examination are recorded. The number of young finally born helps to show how nearly correct the examinations have been.

The records now contain several hundred such examinations and show that absorption of embryos may take place not only during early stages, but after the fetuses have attained considerable size. The difference between absorption and partial abortion may usually be recognized by the fact that the embryo being absorbed may exist for some time as a small lump in the uterus, while the aborted embryo disappears from the uterus and leaves no palpable remains. There are exceptional cases in which the uterus is unusually swollen or congested after the abortion and these on being felt would still seem to contain a partial embryo. The cages of the pregnant females are exam-

ined every morning and afternoon and aborted embryos or placentae are generally located, yet instances do occur of early abortion possibly during the night in which no trace of the aborted material is found, since the female very quickly attempts to eat the aborted products.

Fig. 6 On the right a normal 19-mm. embryo taken from the right horn of the uterus of an alcoholic female. The left horn of the uterus contained the degenerating mass shown on the left which was attached to a small placenta and represents an embryo in the process of being absorbed in utero. The mother had an alcoholized father.

During the two years which supply the data for the present study the females have been very carefully and consistently examined throughout their pregnancies, and the records of absorbed and premature or aborted young are very accurate for all

periods after the embryos are of sufficient size to be detected by this method of external examination. To convey some idea of how accurately one may detect a structure by palpation through the abdominal wall of the guinea-pig, it may be stated that a slightly cystic ovary has frequently been diagnosed by such an examination.

A normally developed embryo 19 mm. crown rump length is shown in figure 6 and near it is seen an amorphous embryonic mass 2 mm. in longest diameter which represents the other member of the litter. The two were in different horns of the uterus. The placenta of the normal embryo was of the usual size, while the one associated with the arrested specimen was only about one-half as large. The entire mass of the smaller ovum in the uterus was about that of a ten-day specimen, while the normal individual was a typical twenty-day specimen. This case was detected by external examination and was merely opened in order to use the embryos for illustrating the phenomenon. In the explanation of the figure the ancestry of the embryos is given.

The intrauterine absorption of embryos, as stated above and indicated in table 1, may occur in normal guinea-pigs. A. W. Meyer ('17) has very recently described the histological conditions found in partially absorbed embryos which he had obtained during a study of the prenatal growth of the guinea-pig. There is considerable data from our study to indicate that this absorption of embryos is somewhat more frequent in the alcoholic than in the normal lines.

7. A COMPARISON OF THE QUALITIES IN THE DIFFERENT GENERATIONS OF THE ALCOHOLIC LINES AS THEY BECOME FURTHER REMOVED FROM THE GENERATION DIRECTLY TREATED

It has been mentioned in discussing the improvement of the records in table 1, as compared with our previous reports, that this advantage is partly due to the larger number of late-generation animals at present included. We may now analyze the alcoholic lines for a comparison of the qualities of the early and

late generations, F_1 to F_4, on the basis of their productivity and mortality records. Such an analysis is of particular importance to test in the first place whether the effects of the alcohol treatment on the germ cells are permanent, altering their qualities in inheritance, and in the second place whether an increasing amount of normal germ plasm acquired with each generation may tend to offset the original alcoholic effect by dilution.

Table 2 contains the data from the non-inbred alcoholic lines divided into different generations. The first vertical column gives the records for 233 control young as a standard of comparison. These are the same records shown in the normal column of table 1, except that in the present table we have included in the first horizontal line under each group the average birth weight of the litters produced. This is termed the average litter weight and is recorded in grams. For the normal stock this average productivity is 197.12 grams; that is, the average weight of all the litters at birth was this amount. The average litter weight is in a way associated with the average litter size, since a litter containing several young though each individual may not be so large, will probably weight more than a litter of fewer or of one young. Thus a group having a higher average litter than another group will also probably have a higher average litter weight, though this is not necessarily the case, as will be seen on comparing the several columns of the table.

The second column contains the alcoholic line animals. This again is the same 594 alcoholic animals shown in the second column of table 1 and is given here for comparison with the four following groups, each of which is a certain portion of this total column. The average productivity for the alcoholic animals is 170 grams, or 37 grams less than the control, and when corrected on the basis of the average litter size, it is 5.6 grams less than it should be according to the normal standard.

The third column gives the records of 186 animals with one or both parents treated with alcohol, the F_1 generation. Thirty-three of these animals also had a slight alcoholic history in their ancestry, and thus the entire group are not pure F_1 alcoholics. The proportion of large and small litters in this column is about

TABLE II

A COMPARISON OF THE RECORDS OF ANIMALS OCCURRING IN THE DIFFERENT GENERATIONS OF THE ALCOHOLIC STOCK

	Normal stock	Alcoholic animals of all generations	Alcoholic animals with treated parents F_1	Alcoholic animals with treated grand-parents, great-grand parents and great-great-grand parents F_{2-3-4}	Alcoholic animals with treated great-grand parents and great-great-grand parents F_{3-4}	Alcoholic animals with treated great-grand parents F_4
Total number	1 2 3 4 5 10 46 90 72 15 24.03% 37.35% Average litter wt. 197.12 gr. Mat.fail. 4(4.54%) 233	1 2 3 4 5 38 162 279 100 15 33.61% 19.32% Average litter 2.47 Average litter wt. 170.09 Mat.fail. 36(13.04%) 594	1 2 3 4 5 9 54 90 28 5 34.61% 17.74% Average litter 2.51 Average litter wt. 165.31gr. Mat.fail. 11(12.94%) 186	1 2 3 4 5 28 106 192 72 10 32.84% 20.09% Average litter 2.47 Average litter wt. 172.21gr Mat.fail. 25(13.15%) 408	1 2 3 4 5 10 26 66 40 5 24.47% 30.61% Average litter wt. 176.78gr Mat.fail. 9(13.84%) 147	1 2 3 4 5 2 4 21 8 0 11.4% 22.85% Average litter 2.69 Average litter wt. 193.53gr Mat.fail. 1(7.14%) 35
Lived over 3 months	1 2 3 4 5 10 39 75 45 12 100% 84% 83% 62.5% 80% 77.68%	1 2 3 4 5 31 132 170 48 2 81.5% 81.48% 60.93% 48% 13.33% 64.47%	1 2 3 4 5 8 44 48 6 0 89.89% 81.48% 53.33% 15% 0% 56.98%	1 2 3 4 5 22 86 123 42 2 78.57% 81.13% 64.06% 58.33% 20% 61.40%	1 2 3 4 5 9 18 48 25 2 90% 69.33% 72.72% 62.5% 40% 69.38%	1 2 3 4 5 2 3 16 8 0 100% 75% 76.09% 100% 82.85%
Absorbed, Premature, Still born	1 2 3 4 5 0 4 7 16 0 (51.92%)	1 2 3 4 5 4 19 79 34 12 (70.14%)	1 2 3 4 5 0 6 32 13 5 (70.0%)	1 2 3 4 5 1 2 14 47 21 7 (69.92%)	1 2 3 4 5 1 7 13 12 2 (71.77%)	1 2 3 4 5 1 0 2 0 0 (33.33%)
Died within 3 months	1 2 3 4 5 0 3 8 11 3 (48.08%)	1 2 3 4 5 3 11 30 18 1 (29.85%)	1 2 3 4 5 1 4 10 9 0 (30.0%)	1 2 3 4 5 1 2 6 22 9 1 (30.08%)	1 2 3 4 5 0 2 5 3 1 (22.22%)	1 2 3 4 5 1 0 3 0 0 (66.66%)
Total dead	1 2 3 4 5 0 7 15 27 3 15.21% 16.66% 37.5% 20% (22.31%) = 100	1 2 3 4 5 7 30 109 52 13 18.41% 18.51% 39.06% 52% 86.66% (35.52%) N 18.79% = 189	1 2 3 4 5 1 10 42 22 5 11.11% 18.5% 46.66% 84.19% 100% (43.01%) N 18.65% = 230	1 2 3 4 5 6 20 69 30 8 21.42% 18.86% 35.93% 41.66% 80% (32.59%) N 18.89% = 172	1 2 3 4 5 1 8 18 15 3 10% 30.76% 27.27% 37.5% 60% (30.61%) N 21.07% = 145	1 2 3 4 5 1 1 5 0 0 25% 23.80% (17.14%) N 26.25% = 84

the same as in the total alcoholic column, 34.6 per cent of the animals were born in litters of less than three and only 17.74 per cent in litters of more than three. The normal record as pointed out before is just about the reverse of this. The average-size litter in which the F_1 animals occur is 2.51, which is slightly larger than for the total alcoholic column, but the average weight of these litters is less than for the entire alcoholic lines, being 165 against 170 grams. As compared with the control the average productivity of this column is 32 grams low, and when corrected on the basis of the average-litter size, the litters are then more than 13 grams less than the control standard. The mating failures are about the average alcoholic result, 12.94 per cent.

The mortality record of the F_1 animals is not so good as for the entire alcoholic group, only 56.98 per cent of them living longer than three months as against 64.47 per cent. The total mortality is 43.01 per cent, and when this is corrected on the basis of the normal mortality for the various-size litters in which the individuals occurred, we find that the F_1 mortality is almost 2.3 times the control record, or 230 against 100. The corrected mortality here as compared with the entire alcoholic group is 230 against 189, or 41 points higher.

The proportion of prenatal to postnatal mortality corresponds closely to that of the entire alcoholic group and contrasts with the control in the same way as discussed in considering table 1.

Finally, then, the F_1 group of animals from either one or both treated parents, are inferior to the alcoholic group as a whole in having a higher mortality record and in occurring in litters of a lower average weight although of equal average size.

The fourth column contains the records of animals more than one generation distant from the alcohol treatment; that is, those having treated grandparents, great-grandparents, or great-great-grandparents, or combinations of these, F_2, F_3, and F_4 generations. All of the alcoholic animals from column 2 are included in this column, except the third column of F_1 animals; there are thus 408 individuals.

The distribution of the animals in large and small litters is closely the same as in the two preceding columns, over 30 per cent being in litters of less than three and 20.09 per cent in litters larger than three.   The average-size litter and the average litter weight are just about what is found for the total alcoholic group and somewhat better than for the F_1 group.   The percentage of surviving animals is a little better than the total alcoholic group and considerably better than the F_1 group.   The prenatal and postnatal mortality proportions follow the typical arrangement for the alcoholic lines, the prenatal being about two and one-third times higher than the postnatal.   The total mortality among these animals is about 10 per cent lower than for the F_1 group and slightly below the record of the total alcoholic lines. When the mortality is corrected in terms of the normal mortality for the different-size litters and stated on the basis of 100 for the control stock, it becomes 172 as against 230 for the F_1 column and 189 for the all generations alcoholic column.

The fifth column records 147 animals still further removed from the treated generation; these had treated great-grandparents or great-great-grandparents or both, the F_3 and F_4 generations.   Some of these animals may have had only one or two alcoholic ancestors out of eight or sixteen; therefore, the proportion of modified to normal germ plasm is often very small.

The arrangement in large and small litters differs from the other alcoholic groups and approaches that shown by the normal lines very closely, there being a higher percentage born in large litters than in small.   The average-size litter is larger than in the three preceding columns, although still well below the control.   The average litter weight is low when compared with the normal lines and only about the same as in the three preceding columns when taken in connection with the average size of the litters.   When corrected for the average size, the weight of the litter falls more than 10 grams below the control record.   The mating failures still show the high percentage of the alcoholic lines, being over three times as many as in the control.

A greater percentage of individuals survived than in any of the preceding groups except the control.

The proportion of prenatal to postnatal mortality shows the arrangement characteristic of the alcoholic groups. As a matter of fact, the prenatal mortality is really unusually high, and this is probably due to the high percentage of large litters, as among these the prenatal mortality is most frequent. It is as though the animals of this group had produced almost as high a proportion of large litters as the control animals and still they were not sufficiently good quality as compared with the control to keep down the prenatal mortality in these high litters.

The total mortality when corrected on the normal rate for the litter sizes and expressed on the basis of 100 for the control becomes 145. This is a decided improvement over the other alcoholic groups, although poor in the light of the control.

From a survey of this column it may be concluded that animals as far as three generations removed from the direct alcohol treatment are still differentiated as a group from the control in regard to the weight of the litters in which they are born, the tendency of the matings to result in failure, the high proportion of prenatal mortality over postnatal, and the total mortality which is one and one-half times higher than the normal. All of these differences exist in spite of the fact that more and more normal germ plasm has been introduced during each generation until some of these animals may have had as many as six or seven normal great-grandparents against one or two treated or alcoholic great-grandparents, though the average of course had somewhat more treated ancestry than this.

One of the F_3 individuals, descended from treated great-grandparents, is shown in figure 7. The animal on the left was a non-inbred female, No. 803, with six of its eight great-grandparents treated with alcohol and only two, on the paternal side, were normal. Its great-grandparents may be written thus: A indicating alcoholic and N normal, the ♀ on the left, in the formulae: [(AxA) (AxA)] [(NxA) (AxN)]. The animal on the right is an ordinary normal guinea-pig born on the same day as the small degenerate specimen which weighed only one-third

Fig. 7   On the left a non-inbred female, No. 803, with six of its eight great-grandparents treated with alcohol and only two on the paternal side not treated. She was small and degenerate and lived only one day.   On the right is shown a normal animal born on the same day, the two being photographed on one plate.

Fig. 8   Two F_3 guinea-pigs born in the same litter from a normal father and a mother derived from four alcoholized grandparents.   The albino female, No. 955, on the left weighed at birth 90 grams, the small defective male on the right weighed only 38 grams and died within two days; the sister is still alive.

as much and lived only one day. Although some young from control parents do die shortly after birth, they are not so unusually small nor degenerate in appearance as the defective young of the alcoholic lines.

Another even more striking example of the small defective animals appearing in the F_3 generation is shown by the photograph, figure 8. The two individuals in this picture were born in the same litter. Their mother was a black and red animal from four alcoholized grandparents and their father was a normal albino male, [(AxA) (AxA)] [N]. The F_3 animal on the left, No. 955, is an albino female weighing at birth 90 grams. She is thus an unusually large animal to be a member of a litter of three and is of the type of the normal albino father. Her small degenerate brother on the right weighed only 38 grams at birth, had a severe tremor which rendered him incapable of normal progressive movements, and he lived only two days. His degeneracy and black and red color are both qualities for which he was indebted to his alcoholic mother. A marked discrepancy in either size or condition between two members of the same litter at birth is entirely lacking among our control lines. It is rarely so decided as this case illustrates, yet very frequent in the alcoholic lines and particularly in the F_2 and F_3 generations. A number of illustrations of this type could be continued to show that the quality of the later generations from alcoholized ancestors is decidedly subnormal.

Such conditions as the above occur not only in spite of the introduction of normal germ plasm which tends to overshadow the alcohol effect, but also in spite of a rather harsh individual selection which is at work tending to improve the stock with each generation. Almost all of the badly defective individuals in the alcoholic lines are lost early in their career, as is shown by the high prenatal mortality; other less defective ones die soon after birth, such as those pictured above, and only the best live to become fertile adults. It is thus found that even this selected group mated with many normal individuals still possesses enough of the modified germ plasm which resulted from the early alcohol treatment to cause their offspring to be inferior to

the control animals in a number of important qualities that render them less capable of survival.

These two factors, the constant introduction of more normal germ plasm and the elimination of all the weaker alcoholic individuals so that only the stronger reproduce, may finally in late generations so purify the alcoholic lines as to cause them to attain a condition equally as good as the normal.

The sixth and last column of table 2 may illustrate such a condition, though it contains the records from only a few animals. These animals are descended from one or more treated great-great-grandparents, the F_4 generation. They are four generations removed from the alcoholic treatment.

The average-size litter is almost as large as in the control, and on the basis of its size it is actually heavier than the control average. It may be said from the evidence shown that the productivity here is equally as high as in the control.

A higher percentage of individuals survived than among the control, and even though the mortality figures are small there was certainly no tendency toward a high prenatal mortality. On the contrary, there was scarcely any prenatal mortality, so that the record in no way resembles that of the alcoholic lines. On the basis of 100 for normal stock mortality, the mortality here corrected for litter size is only 84, or 16 per cent better than the normal. It is actually in the table 5 per cent lower than the control.

After having considered the last column, the F_4 animals with their very good record, it should be recognized that these same animals are included with the F_2 and F_3 individuals in the fourth and fifth columns. Their presence in these columns, particularly in the fifth, has tended to incline the records toward the normal. One must realize, therefore, that the F_2 and F_3 animals if considered alone would present even stronger alcoholic records than are indicated in the fourth and fifth columns.

The table shows that the nearer to the direct alcohol treatment an animal is produced, the more inferior in quality it will be as a result of the high amount of modified germ plasm contained in the germ-cell complex from which it arises. Therefore, the records

of F_1 individuals are worse than the records of the sum of all alcoholic generations, as is seen on comparing the third column with the second. The later generations being further and further removed from the treatment and having less and less modified germ plasm on account of the constant introduction of normal stock are progressively improved until finally the F_4 generation has its modified germ plasm diluted to such a degree that its record is on par with the control.

The ancestors of these late-generation animals were also successively selected from the least affected of the alcoholic stock, being those animals capable of survival and reproduction, while the most highly affected died or were sterile and incapable of reproduction. We assume the probability that the more nearly normal animals with stronger bodies also carry germ cells that are less affected than those in the more degenerate individuals. Most of the grossly defective individuals which reach maturity are sterile as evidence in this direction. Thus individual selection being in this case a selection of germ plasm as well as soma, helps materially to improve the quality of the later generations.

8. A COMPARISON OF ANIMALS FROM DIRECTLY TREATED FATHERS AND FATHERS OF ALCOHOLIC STOCK WITH ANIMALS FROM DIRECTLY TREATED MOTHERS AND MOTHERS OF ALCOHOLIC STOCK AND WITH OTHERS FROM BOTH PARENTS OF ALCOHOLIC STOCK

Are the general conditions induced by directly treating the father with alcohol the same as those resulting from treating the mother, and are they equal in extent? Do fathers of alcoholic ancestry beget offspring of better or worse quality than offspring produced by mothers of similar alcoholic ancestry? Or are the effects of the alcohol treatment on the germ cells, which is expressed through several generations, carried with equal degree by both the alcoholic father and the alcoholic mother? We shall attempt in this and the following section to supply data which may serve to partially, at least, satisfy these queries as well as furnish an analysis of several other more detailed propositions.

TABLE III

AN ANALYSIS OF THE EFFECTS ON THE PROGENY WHEN ONLY FATHER IS ALCOHOLIC, MOTHER ALCOHOLIC OR BOTH PARENTS ALCOHOLIC

	Alcoholic animals with treated parents (first generation)			Alcoholic animals with parents of alcoholic descent, but not directly treated (other generations, except first)			All animals of alcoholic descent (all generations, first included)		
	Only father treated	Only mother treated	Both parents treated	Only father alcoholic	Only mother alcoholic	Both parents alcoholic	Only father alcoholic	Only mother alcoholic	Both parents alcoholic
Total number	1 2 3 4 5 6 18 24 12 0 40% 20% Average litter 2·30 Aver. product 170·58 Mat. fail 8 (23·52%) =60	1 2 3 4 5 1 18 51 16 0 20·65% 17·39% Average litter 2·78 Aver. product 171·57 Mat. fail 2 (5·71%) =92	1 2 3 4 5 0 0 0 0 0 =1	1 2 3 4 5 6 16 12 20 5 37·28% 43·57% Average litter 2·45 Average product 170·95 Mat fail 6 (20·0%) =59	1 2 3 4 5 7 38 108 24 5 24·72% 15·93% Average litter 2·63 Aver product 176·95 Mat fail 14 (16·84%) =182	1 2 3 4 5 15 52 72 29 0 40·11% 16·16% Average litter 2·31 Aver Product 168·82 Mat fail 6 (7·64%) =167	1 2 3 4 5 12 34 36 32 5 39·64% 30·51% Average litter 2·41 Aver. Product 171·09 Mat fail 14 (13·3%) =121	1 2 3 4 5 8 56 165 40 5 23·35% 16·42% Average litter 2·69 Aver. product 175·14 Mat fail 16 (13·30%) =214	1 2 3 4 5 18 70 78 28 5 44·31% 16·53% Average litter 2·28 Aver product 165·96 Mat fail 6 (6·45%) =199
Lived over 3 months	1 2 3 4 5 6 16 17 1 0 (66·66%)	1 2 3 4 5 1 14 25 5 0 (48·91%)	1 2 3 4 5 0 0 0 0 0 1	1 2 3 4 5 4 12 11 10 0 (62·71%)	1 2 3 4 5 6 30 73 11 2 (67·03%)	1 2 3 4 5 12 44 39 21 0 (69·46%)	1 2 3 4 5 10 30 28 11 0 (65·28%)	1 2 3 4 5 7 44 98 16 2 (60·94%)	1 2 3 4 5 14 58 44 21 0 (68·84%)
Absorbed, Premature, Stillborn	1 2 3 4 5 0 1 6 5 0 (60%)	1 2 3 4 5 0 2 15 8 0 (14·46%)	1 2 3 4 5 0 0 0 0 0	1 2 3 4 5 2 4 1 5 5 (77·27%)	1 2 3 4 5 1 5 25 12 2 (15%)	1 2 3 4 5 1 5 21 4 0 (60·18%)	1 2 3 4 5 2 5 7 16 5 (69·04%)	1 2 3 4 5 1 7 50 20 2 (14·76%)	1 2 3 4 5 1 7 22 4 5 (62·90%)
Died within 3 months	1 2 3 4 5 0 1 1 6 0 (40%)	1 2 3 4 5 0 2 7 3 0 (25·53%)	1 2 3 4 5 0 0 0 0 0	1 2 3 4 5 0 0 0 5 0 (22·72%)	1 2 3 4 5 0 3 10 1 1 (25%)	1 2 3 4 5 2 3 12 3 0 (35·21%)	1 2 3 4 5 0 1 1 11 0 (30·95%)	1 2 3 4 5 0 5 17 4 1 (25·23%)	1 2 3 4 5 3 5 12 3 0 (27·01%)
Total dead	1 2 3 4 5 0 2 7 11 0 (33·33%) N 18·66% =113	1 2 3 4 5 0 4 32 11 0 (51·08%) N 18·15% =281	1 2 3 4 5 0 0 0 0 0	1 2 3 4 5 2 4 1 10 5 (37·28%) N 21·89% =170	1 2 3 4 5 1 9 35 13 3 (32·96%) N 18·54% =171	1 2 3 4 5 3 8 33 7 0 (39·82%) N 18·20% =167	1 2 3 4 5 2 6 8 21 0 (34·71%) N 20·21% =171	1 2 3 4 5 1 12 60 24 3 (39·05%) N 18·94% =206	1 2 3 4 5 4 12 34 7 5 (31·15%) N 17·65% =176

Table 3 is an arrangement of the records of animals on the basis of paternal and maternal alcoholism. The first group of animals are those from parents treated directly and having no other alcoholic history. Thus the total 153 differs from the total 186 animals with treated parents in the third column of table 2, since in thirty-three cases the former group had not only treated parents, but also treated ancestors. The second group contains all the animals with parents of alcoholic descent, but not directly treated, the total number is 408; these are the same animals that compose the fourth column of table 2. The third or last group contains all of the 594 non-inbred animals of the alcoholic lines.

The individuals in each of the three groups are separated into three classes. The classes of the first group are those with only father treated, those with only mother treated, and those with both parents treated. In the second group the young are classified as those from only father alcoholic, which means the father was descended from treated ancestors which may have been either treated males or females. In other words, this is not the record of a pure alcoholic male line, but merely the alcoholic effects, if any, that reach the recorded individual through an alcoholic father regardless of the origin of his alcoholism. The second column of this group shows the records of animals from alcoholic mothers. Here again the mother's alcoholism may be due to treatment of any of her ancestors, male or female. It is not a purely female alcoholic line, but a maternal alcoholic line. The third column of the second group shows records of animals from parents both of which were alcoholic.

In the entire second group the alcoholism of the parents is ancestral, not being due to direct treatment, while in the third group the alcoholism is either direct, ancestral, or both. The third group is, therefore, an arrangement of all the animals from alcoholic lines for a comparison of the influences of maternal and paternal alcoholism.

In the first column of table 3 it is seen that when the father only is treated the results contrast decidedly with the control.

There is a high percentage of small litters and a low percentage of large litters, thus giving next to the lowest average litter contained in all the records, only 2.30 against 2.77 for the normal. The average litter weight is very low on account of the small average litter size. When this is corrected for the proportion of weight to number of individuals in the control litters these small litters from the treated fathers weigh more for their size than do the control, being over 6 grams heavier. This is not an actual advantage since the majority of young born in small litters of one and two are larger than those born in high litters of four or five. The percentage of mating failures is unusually high, 23.52 per cent against only 4.54 per cent in the control. All of these facts would seem to indicate that the treatment of the fathers had evidently lowered their productivity or fertility, causing them to fail to sire offspring in almost one-quarter of the matings and to beget unusually small litters in the other three-quarters of the cases. There must have also been a high 'early prenatal mortality' in view of the remarkably great percentage of small litters and high percentage of mating failures. We must necessarily divide the mortality into prenatal and postnatal, and the prenatal again into 'early prenatal,' as indicated by the small average size litter and high number of mating failures, and 'late prenatal' based on the exact observations of absorptions, abortions, and still births.

Two-thirds of the offspring from treated fathers survived against over three-fourths from the control. The prenatal mortality is a larger proportion of the total than in the normal. The total mortality when corrected to the normal rate for the different-size litters in which the animals were born is 178 in terms of the control as 100. This is only slightly below the mortality rate of 189 for the entire non-inbred alcoholic group.

When the mother alone was treated the records of the offspring differ considerably from the above. The percentage of small litters is only slightly higher than the percentage of large litters, and the average-size litter, 2.78, is as large as the normal. There are very few mating failures, in this regard again

almost a normal record. The productivity of these treated mothers is high and the size of the litters would indicate a very low 'early prenatal mortality.' Here, however, their good records stop.

Although the litters contained as many individuals as the control litters, their average weight was 26 grams below the normal. The large litters from treated mothers actually weighed only as much as the very small litters from treated fathers; therefore, the individual members of the litters from treated mothers were unusually small animals. The 'late prenatal mortality' was proportionately very high—three times the postnatal. Thus many of the young died in utero or were still-born, and those that were born alive were small specimens. The total mortality was 51.08 per cent, corrected for the litter sizes, and expressed in terms of the control as 100 it becomes 281—the highest mortality on record.

We see from the table that treating the mother with alcohol does not appreciably affect her productivity, but greatly depreciates the quality of offspring to which she gives rise. While in the case of the alcoholic father the productivity is greatly reduced, and although the quality of offspring which he begets does not compare favorably with the control, it is considerably superior to that from the treated mother. In the treated mother the alcohol may act not alone on the ova or germ cells, but on the developing embryo as well, while in the father it acts, of course, on the germ cells alone. Does the difference between the qualities of the offspring from these two cases represent the action of the treatment on the developing young in utero? Further, does the reduced productivity on the part of the treated male indicate that the spermatozoön or male germ cells are more sensitive to the treatment than the egg? The remaining columns of this and the following table may throw some light on these questions.

During the period of the experiments now under consideration practically no matings between treated males and females have been made, as the third column of this group shows.

The next group in table 3 are animals derived from parents of alcoholic descent which had not themselves been treated. These are the same 408 animals recorded in the fourth column of table 2. The first class in this group are animals obtained from fathers of alcoholic ancestry and normal mothers; the second class are from mothers of alcoholic ancestry and normal fathers, and the third class are animals produced by two alcoholic parents. As mentioned above, the alcoholic father or mother may owe their condition to either male or female or to both male and female ancestors. These are not purely male or female alcoholic lines such as will be found in the next table.

A comparison of these three columns with the normal records shows clearly the alcohol effects, though not so strongly expressed as when the father or mother is directly treated. The father and mother columns of this group differ very little from one another, which is in marked contrast to the striking differences when the fathers and mothers are directly treated, as seen in columns 1 and 2. In the present columns all of the modified conditions are due to an injury of the germ cells in the treated ancestral generations. This is equally as true of the alcoholic-mother column as of the alcoholic father. For example, the mortality records in the alcoholic father and mother columns are about the same, while there is a remarkable discrepancy between the mortality records of young from treated fathers and treated mothers in the first two columns. The extremely high mortality, largely late prenatal, among the offspring of directly treated females is to some extent due to the direct action of the alcohol upon the early developing embryo in utero. If this action could be eliminated the treated father and mother columns of the first group might become as nearly similar as the alcoholic father and mother columns of the second group. The individuals in the latter two columns are on an average about the same distance removed from the ancestral alcohol treatment, and, therefore, the records would be little affected by a correction on the basis of the generations treated.

When both parents are from alcoholic ancestry the productivity is considerably lowered as shown in the third column by

the high percentage of small litters and low percentage of large litters and consequently the very low average litter of 2.31. This is likely due to the male partner in the combination, as the preceding columns would suggest. The average litter weight, however, is high, so that the individual members of the litter are as heavy as the normal; this, again, may be due to the male influence as expressed in the high early prenatal mortality.

The mortality records, though markedly inferior to the normal, show an advantage over the two previous columns. There is probably a high 'early prenatal mortality' as indicated by the low average litter, but the 'late prenatal mortality' is lower than in any of the foregoing columns except that of the treated fathers, where again the litter was very small and the probable 'early prenatal mortality' high. This close association between the small litters and the low late prenatal mortality makes it seem all the more probable that the litter size is associated with an 'early prenatal mortality' that occurs so near the beginning of development that it cannot be directly observed. On the other hand, this result could be interpreted as due to a lowered fertility. If this were brought about through an elimination of the weaker germ cells we might except also the associated low late prenatal and postnatal mortality, and would have a condition in exact accord with Pearl's interpretation of the results on fowls. We should be glad to accept such an explanation, but for the considerable amount of evidence in our records which points towards a high 'early prenatal mortality' rather more than infertility as the underlying cause of the small litters and low late mortality. It must also be remembered that the infertility among the fowls was found in the females as well as the males, while here it would be confined to the males only.

The slight advantages which appear in favor of the records from both parents alcoholic as compared with records from alcoholic mothers or fathers are due largely to the distance from the treatment of the generations concerned. In the majority of cases the generations are more remote in the both-parent column than in either the father or mother column, and on the basis of

the evidence shown in table 2 this may readily explain the apparent advantages.

The last three columns show the results of the first two groups combined and in addition contain a few records from mixed cases that could not be properly included in any of the previous classes; for example, animals with one parent of alcoholic ancestry and the other parent directly treated, etc.

Here again there is considerable contrast between the alcoholic-father and the alcoholic-mother columns, these differences being due to the influence on the totals of the F_1 records from the treated-father and treated-mother columns of the first group. The productivity when only the father is alcoholic is low, the litters being small and over 21 per cent of the matings result in failure.   It may be inferred that there was a rather high 'early prenatal mortality.'   The average litter weight, however, was about as good as normal.   The late prenatal and postnatal mortality records are better than those from the alcoholic mothers.

The average-size litters from the alcoholic mothers was rather large and the mating failures were much less frequent than from the alcoholic fathers, indicating a lower probable 'early prenatal mortality.'   The average litter weight was lower than from alcoholic fathers, taking into account the size of the litters in the two classes.   The total mortality from alcoholic mothers was high and the proportion of late prenatal to postnatal was excessive.   It is thus seen that a high prenatal mortality is followed by a low postnatal death rate, and this is in accord with our assumption that a high 'early prenatal mortality' will be followed by not only a low postnatal, but also a low late prenatal mortality.   In other words, the more thorough the elimination of defective embryos and fetuses the greater the probability of survival for the selected few that remains to be born.

The last column with both parents alcoholic has a mortality record as good as the alcoholic-father column and better than the alcoholic-mother, but this is only apparent and not real. The column contains only one individual from directly treated parents, and consequently the alcoholic treatment was applied

on the average to more remote generations than was the case in the two single alcoholic-parent columns.

In spite of the generations concerned, there is a higher per cent of small litters and a lower per cent of large litters here than in any other class in the entire table. Consequently there is also the lowest average litter. In so extreme a case there was no doubt a high early prenatal mortality. The average litter weight is actually low, but allowing for the small-size litter the average birth weight of the individuals is about as much as the control, again indicating that an individual selection has occurred through an elimination of the weaker embryos during the early developmental stages.

The extremely small-size litter and the high 'early prenatal mortality' may also in addition to the generations concerned explain to some extent the relatively low total mortality and especially the lower rate of late prenatal mortality as compared with postnatal.

The questions involved in the present section may be still further analyzed by rearranging the data on the basis of only male ancestors treated or only female ancestors treated instead of only father alcoholic and only mother alcoholic. Table 4 presenting this arrangement will be reviewed in the following section, after which several points of interest may be better discussed.

9. A COMPARISON OF LINES FROM ONLY MALE ANCESTORS ALCO-HOLIC WITH LINES FROM ONLY FEMALE ANCESTORS ALCOHOLIC AND WITH THOSE FROM BOTH MALE AND FEMALE ANCESTORS ALCOHOLIC

The records tabulated on the basis of male or female ancestors treated supplement the arrangements in table 3, where the groups are classed for only father or mother alcoholic. In table 3 the alcoholic father may owe his alcoholism to the treatment of any of his ancestors, either male or female or both. The alcoholic effects, if any, are there due to the paternal ancestry. The same applies to the groups with only mother alcoholic.

In table 4, on the other hand, the groups with only male ancestors treated owe their modified conditions, if such exist, entirely to the effects of the treatment on male animals, though the individual being considered may have inherited this alcoholic effect through its mother. Thus animals in the columns with only male ancestors treated were not necessarily derived from alcoholic fathers, but may have been produced by alcoholic mothers which, however, owe their alcoholic condition to one or more treated male ancestors. The table permits a comparison of the action of the treatment on the male germ cells and the transmission of the effects with the action of the alcohol treatment on the female germ cells and the effects transmitted to the different generations. While the last table permitted a comparison of the animals derived from males of alcoholic stock with others derived from females of alcoholic stock. The two tables serve to analyze very completely the problem of the parts played by the sexes in the acquisition and transmission of the effects of the alcohol treatments.

The three columns in the first group of table 4 are the same as those of the first group of table 3, being the records of F_1 animals derived from treated fathers, which have only one male ancestor treated according to the table 4 arrangement, and F_1 animals derived from treated mothers or from only the one female ancestor treated. This group was discussed in reviewing the third table. The points of chief interest in the present connection are the decidedly inferior conditions of the offspring from the treated females as compared with those from the treated males, in so far as their measured mortality records and birth weights per litter are concerned. On the other hand, the records from treated males suffer as regards the 'early prenatal mortality' indicated by the small average-size litter and the high percentage of mating failures, while the records of the treated females in regard to these conditions are equally as good as those of the control animals.

The next three columns of table 4 are highly important, since they contain the results of matings when, first, only male ancestors are treated; second, when only female ancestors are

TABLE IV

AN ANALYSIS OF THE EFFECTS ON THE PROGENY WHEN ONLY MALE ANCESTORS WERE TREATED, FEMALE ANCESTORS TREATED OR BOTH MALE & FEMALE ANCESTORS TREATED

	Alcoholic animals with treated parents (first generation)			Alcoholic animals with parents of alcoholic descent, but not directly treated (Other generations, except first)			All animals of alcoholic descent (all generations, first included)		
	Only father treated	Only mother treated	Both parents treated	Only ♂ ancestors treated	Only ♀ ancestors treated	♂ & ♀ ancestors treated	Only ♂ ancestors treated	Only ♀ ancestors treated	♂ & ♀ ancestors treated
Total number	1 2 3 4 5 / 6 18 24 12 0 / 40% 20% / Average litter 2.30 / Average product 170.38 / Mat. fail 8(13.52%) / 60	1 2 3 4 5 / 1 18 51 16 0 / 20.65% 17.39% / Aver. litter 2.78 / Aver. product 171.57 / Mat. fail.2(5.71%) / 92	1 2 3 4 5 / 0 0 0 0 0 / 1	1 2 3 4 5 / 10 38 63 24 10 / 33.10% 23.51% / Aver. litter 2.50 / Aver. product 173.25 / Mat. fail.14(14.44%) / 145	1 2 3 4 5 / 4 18 48 12 0 / 26.82% 14.63% / Aver. litter 2.56 / Aver. Product 175.51 / Mat. fail.11(13.02%) / 82	1 2 3 4 5 / 14 50 81 36 0 / 35.3% 19.33% / Aver. litter 2.41 / Aver. product 170.73 / Mat. fail 11(12.77%) / 181	1 2 3 4 5 / 18 64 90 36 15 / 36.77% 22.86% / Aver. litter 2.42 / Aver. product 168.81 / Mat. fail 22(11.24%) / 223	1 2 3 4 5 / 5 40 108 28 0 / 24.86% 15.46% / Aver. litter 2.66 / Aver. Prod 171.27 / Mat. fail 3(4.22%) / 181	1 2 3 4 5 / 15 58 81 36 0 / 28.42% 19.44% / Aver. litter 2.37 / Aver. prod 170.17 / Mat. fail.11(10.01%) / 190
Lived over 3 months	1 2 3 4 5 / 6 16 17 1 0 / (64.46%)	1 2 3 4 5 / 1 19 25 5 0 / (47.91%)	1 2 3 4 5 / 1 0	1 2 3 4 5 / 6 31 43 9 2 / (63.15%)	1 2 3 4 5 / 3 14 35 7 0 / (71.95%)	1 2 3 4 5 / 13 54 62 10 2 / (69.06%)	1 2 3 4 5 / 13 54 62 10 2 / (63.22%)	1 2 3 4 5 / 4 29 63 12 0 / (54.66%)	1 2 3 4 5 / 14 49 45 26 0 / (70.52%)
Absorbed, Premature, Stillborn	1 2 3 4 5 / 0 1 16 5 0 / (60%)	1 2 3 4 5 / 0 2 25 8 0 / (74.46%)	1 2 3 4 5 / 0 0 0 0 0	1 2 3 4 5 / 2 6 16 12 7 / (79.62%)	1 2 3 4 5 / 1 0 9 4 0 / (60.86%)	1 2 3 4 5 / 1 8 22 5 0 / (64.28%)	1 2 3 4 5 / 2 8 23 17 12 / (75.60%)	1 2 3 4 5 / 1 3 34 12 0 / (68.44%)	1 2 3 4 5 / 1 3 22 5 0 / (64.28%)
Died within 3 months	1 2 3 4 5 / 0 1 1 6 0 / (40%)	1 2 3 4 5 / 0 2 7 3 0 / (25.53%)	1 2 3 4 5 / 0 0 0 0 0	1 2 3 4 5 / 2 1 4 3 1 / (20.31%)	1 2 3 4 5 / 0 4 1 1 0 / (39.13%)	1 2 3 4 5 / 0 1 14 5 0 / (35.71%)	1 2 3 4 5 / 3 2 5 4 1 / (24.40%)	1 2 3 4 5 / 0 9 11 4 0 / (31.50%)	1 2 3 4 5 / 0 1 14 5 0 / (35.71%)
Total dead	1 2 3 4 5 / 0 2 7 11 0 / (33.33%) N 19.66% / = 178	1 2 3 4 5 / 0 4 32 11 0 / (51.08%) N 13.15% / = 231	1 2 3 4 5 / 0 0	1 2 3 4 5 / 4 7 20 15 8 / (37.24%) N 19.75% / = 193	1 2 3 4 5 / 1 4 13 5 0 / (28.04%) N 18.54% / = 152	1 2 3 4 5 / 1 2 36 10 0 / (30.93%) N 19.11% / = 161	1 2 3 4 5 / 5 10 28 26 13 / (36.77%) N 19.52% / = 193	1 2 3 4 5 / 1 11 45 16 0 / (40.33%) N 19.04% / = 211	1 2 3 4 5 / 1 4 36 10 0 / (24.47%) N 13.84% / = 166

treated, and third, when both male and female ancestors are treated with all first generation, F_1 offspring, excluded. The modified conditions shown by these records are due to an hereditary transmission of the defects and not in any case to the direct influence of the treatment on the developing animals.

The fourth column from only male ancestors when compared with the normal stock in tables 1 and 2 shows a higher 'early prenatal mortality' based on the average litter size and high number of mating failures, a lower average litter weight, a higher late prenatal mortality, and a higher total mortality. The results of these matings are, therefore, from any point of view worse than the results of normal matings. And they prove the hereditary transmission of the defects arising from the treatment of the male animals.

The same can be said for the female column, the results shown here also being worse than from the normal matings. The 'early prenatal mortality' is higher, the average litter weight, indicating the total productivity is smaller, the late prenatal and total mortality are higher, while the mating failures are about the same as in the control records. Therefore, the treatment of female individuals also induces effects that are transmitted to later generations through the germ cells.

When, however, the records of the fourth and fifth columns are compared, it is found that the treatment of male ancestors gives in every point considered more marked effects on the qualities of the descendants than the treatment of female ancestors. Among the descendants of treated males there is a higher early and late prenatal mortality, a decidedly higher total mortality, and more mating failures than among those from treated female ancestors, while the first and second columns show the opposite to prevail so far as litter weight and mortality are concerned for first-generation, F_1, animals from directly treated males and females. These inferior results, so far as late prenatal and total mortality are concerned on the part of the offspring from the directly treated female, may be interpreted as due to the direct influence of the treatment upon the young in utero. On the other hand, the improved records from the

treated-female line during later generations can probably be explained in part by the higher mortality of the offspring in the first generation, thus bringing about a greater elimination of the weaker individuals. In other words, these animals from only female ancestors treated have withstood a somewhat more severe selection during the first generation than have the offspring from only treated male ancestors.

As a final possibility it must be recognized that the superior records of the late generations descended from treated female ancestors as compared with the records of similar generations descended from treated males, may be due to a smaller influence of the alcohol treatment on the ova, or female germ cells, than on the spermatozoa.

The sixth column from treated male and female ancestors shows, in comparison with the two preceding columns, the highest 'early prenatal mortality' based on the many small-size litters. There is also the lowest average litter weight. The late prenatal mortality, total mortality, and mating failures, while higher than for the treated-female line, are lower than in the treated-male line. The complete absence of matings between directly treated animals as seen in the third column of this table makes comparisons and explanations of the results in this sixth column very difficult.

The last three columns of the table show the combined results from all generations. The column for both male and female ancestors treated shows the highest early prenatal mortality, the male treated line the highest late prenatal mortality, and the female treated line the highest postnatal mortality.

In general it may be stated after reviewing this and the foregoing table that the treatment of males produces in their descendants a high early mortality, especially early prenatal. The treatment of females produces in their descendants a high later mortality, especially late prenatal and postnatal. The treatment of male and female ancestors produces in their descendants the highest early prenatal mortality, but the lowest late prenatal and postnatal mortality.

In table 4, male and female ancestors treated does not necessarily indicate that all records in the column were derived from matings between two alcoholic parents, since both males and females may have been treated among either the ancestors of the mother or the father, but not necessarily both.   For this reason the sixth and ninth columns of table 3, in which both parents were in all cases from alcoholic ancestry, show more decidedly that two alcoholic parents when mated together give the very highest early prenatal mortality, but a low late prenatal and postnatal mortality.   This last conclusion is extremely interesting in connection with Pearl's results on fowls.

Pearl found that when two alcoholic fowls were mated together, the percentage of infertile eggs was higher than from any other combination, while the prenatal mortality, embryos dying in shell, and the postnatal mortality were the lowest. This is exactly what the guinea-pig records show, provided our 'early prenatal mortality' (indicated by the small litter size, the frequent mating failures, and the observed mortality occurring in utero during all later stages of development) can be considered the same as many of Pearl's 'infertile eggs.' Without intending any adverse criticism of the designation 'infertile,' we may again suggest the possibility that a certain proportion of these eggs had really begun development, but had died in the early cleavage or gastrular stages, and yet on examination, other than a minute microscopic study, they appeared as infertile or unfertilized eggs.   If this were true, they could be classed in the early prenatal mortality records.   Such an adjustment would serve to harmonize the fowl and guinea-pig records in another important respect.

Pearl has attributed the good qualities of the offspring from his alcoholic parents to a germinal selection which has tended to cause all weak germ cells to be completely put out of commission by the alcohol treatment and only the very best have survived to produce embryos, and these therefore show a low percentage of deaths in shell and a low postnatal mortality.   A selection is also playing its rôle in the case of the guinea-pigs, but here it is not acting alone on the germ cells, but more evi-

dently on the developing individuals. The selection in our case is a continuous selection of individuals, eliminating, no doubt, certain of the least resistant germ cells, but continuing to act on the embryonic population to eliminate the most defective of these during very early developmental stages, and so on until the individuals born are a mixture of strong specimen and others only sufficiently strong to have reached birth and possibly to survive in a subnormal fashion for a shorter or longer time. This continuous, both germinal and individual, selection seems to us more to be expected than the abruptly broken germinal selection advocated by Pearl, which completely eliminates all weak germs, and therefore no weak individuals begin development. We must admit that the data from Pearl's double alcoholic matings considered alone strongly suggest only a germinal selection, but the results from our double alcoholic matings, while leaning in the same direction, still show a greater late prenatal and postnatal mortality than do the control matings, and in addition present much evidence to suggest a very high early embryonic elimination. This same early embryonic elimination may be included among the high percentage of infertile eggs resulting from the matings of two alcoholic fowls, and in the case of the fowls it may be so much more severe that the later mortality records compare favorably with the control. This again would lead us to an abrupt break after the high very early prenatal mortality and might be thought to vitiate our entire supposition, yet the guinea-pig records show almost all gradations up to the condition for the fowls.

Our results show that in the alcoholic lines the higher the early prenatal mortality and consequently the smaller the average-size litter, the lower the late prenatal and postnatal death rate, much as Pearl also finds for fowls. These findings will be still further discussed in connection with the sex ratio, table 6.

10. TREATING MALES WITH ALCOHOL FOR ONE AND TWO GENERA-
TIONS AGAINST TREATING FEMALES FOR ONE AND
TWO GENERATIONS

An experiment has now been in progress for some time in which straight male lines have been treated with alcohol for several generations in order to compare the results with those from the treatment of straight female lines for several generations. That is, the original males are treated, their sons are then directetly treated, their grandsons, great-grandsons, and so on; these we consider the straight male lines. The treated females, their directly treated daughters, granddaughters, and so on constitute the straight female lines. There are now a few third- and fourth-generation individuals, though not a sufficient number to tabulate. We shall thus for the present confine our attention to the records from the originally selected and treated males and females and the treated sons of these males and daughters of the females.

The records are arranged in table 5. In considering the table it must be stated that the original animals in this experiment have been carefully selected large strong specimens that were particularly good breeders. Such a choice has been made on account of the severity of the treatment to which the descendants are to be subjected through a number of generations. Only the best animals are likely to produce descendants sufficiently strong to be treated with alcohol and to continue to reproduce for one generation after another. The fact that such a selection is possible does not reflect on the general population, since no population is so perfect that certain individuals are not better than others. This selection probably accounts for the presence in all the groups of table 5 of some offspring of unusually large size.

The pedigrees or conditions of the animals in the different generations are expressed by the following symbols or formulae. A normal animal is represented by the letter N and one treated with alcohol by A. The symbol for the male is placed to the right of that for the female. Thus the first column NN are the normal control animals for comparison, the second column NA

TABLE V
MALES TREATED WITH ALCOHOL FOR TWO GENERATIONS COMPARED WITH FEMALES TREATED FOR TWO GENERATIONS

	NN					NA					N $\frac{NA}{A}$					AN					$\frac{AN}{A}$ N -				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Total number	10	46	90	72	15	3	6	6	8	0	2	12	15	4	0	14	30	12	0	0	2	21	4	0
	24.05% 84.78% 85.33% 62.50% 80%				37.33%	39.13% 100% 83.33%				34.75%	42.42% 83.33% 60% 25%				12.12%	26.31% 73.51% 50% 25%				21.05%	8.69% 50% 33.33%				2 (2.0%)
	Average litter 2.71 Mat.fail. 4 (4.94%) =253					Average litter 2.30 Mat.fail. 3 (2.3.07%) 23					Average litter 2.35 Mat.fail. 5 (26.31%) 33					Average litter 2.71 Mat.fail. 0				57	Average litter 2.87 Mat.fail. 2 (2.0%) 23				
Lived over 3 months	10	39	75	45	12	3	6	5	0	0	2	10	9	1	0	2	11	15	3	0	2	7	0	0
	100% 84.78% 83.33% 62.50% 80%					100% 83.33%					100% 83.33% 60% 25%					100% 73.51% 50% 25%					50% 33.33%				
	(71.68%)					(60.86%)					(66.66%)					(52.63%)					(34.78%)				
Absorbed, Premature, Stillborn	0	4	7	16	0	0	0	0	5	0	0	1	6	0	0	0	2	11	8	0	0	13	0	0
	(51.92%)					(55.55%)					(63.63%)					(77.77%)					(86.66%)				
Died within 3 months	0	3	8	11	3	0	0	1	3	0	0	3	0	3	0	0	4	1	0	0	0	1	0	0
	(48.08%)					(44.44%)					(36.36%)					(22.22%)					(13.33%)				
Total dead	0	7	15	27	3	0	0	1	8	0	0	2	6	3	0	0	15	9	0	0	0	14	0	0
	15.21% 16.66% 37.5% 20%					16.66% 100%					16.66% 40% 75%					21.42% 50% 75%					50% 66.66%				
	(22.31%) = 100					(39.13%) N 21.56% =181					(33.33%) N 17.60% =189					(47.36%) N 20.35% =232					(65.21%) N 16.47% =395				
Defective	0	2	0	0	0	0	2	0	0	0	0	2	0	0	0	0	2	4	0	0	1	2	0	0
											6.66% 25%					7.14% 13.33% 8.33%					4.16%				
											(6.06%)					(10.52%)					(4.34%)				
Oversized (more than 500g. when 3 months old)	1	2	5	4	0	1	1	3	0	0	1	2	3	0	0	1	2	0	0	0	2	3	4	0
	30% 10.86% 5.55%					33.33% 16.66%					8.33% 6.66%					7.14%					4.76%				
	(5.57%)					(8.69%)					(6.06%)					(1.75%)					(4.34%)				
Undersized (less than 300g. when 3 months old)	0	1	3	0	0	0	2	0	0	0	0	2	3	0	0	0	2	3	0	0	0	2	3	0
	2.17%																								
	(0.42%)																								

show records of offspring derived from a treated father A and a normal mother N. The third column are offspring from treated males which were also derived from treated fathers and normal mothers, $\frac{NA}{A}$, mated with normal females, N. The next straight male generation treated and paired with normal females would be expressed by $\frac{N\frac{NA}{A}}{N\frac{}{A}}$, the offspring from such a combination would have had their father, a grandfather, and a great-grandfather treated with alcohol and their mother, grandmothers, and great-grandmothers all normal, and so on for later generations. Animals of these higher pedigrees will be recorded in a future communication. In the table 5 only records from treated fathers are given in column 2 and from treated father and grandfather in column 3. The fourth column shows records from normal fathers and treated mothers, AN, and the fifth column from normal fathers mated with treated females which were derived from treated mothers, $\frac{AN}{A}$ N.

The numbers in all of the columns are rather small, but in every case the records differ from the control. There is a remarkable similarity between the two treated-male groups and also between the two treated-female groups, but a striking contrast exists between the male records as a class and the female records.

In the two male columns the average litter is very small and the mating failures high. The percentage of surviving young, though well under the control record, is equally above the female records. The corrected total mortality in both columns is over 180 against 100 for the control. The proportion of late prenatal to postnatal mortality is slightly contrasted in the one treated male generation column, but more so in the two treated generations column. There are no defective animals in the NA column, but a small per cent of such are seen in the $N\frac{NA}{A}$ group.

The average litter size is high in both female columns and the mating failures lower than in the male groups. While the total mortality is extremely high, being in the two-generation treated column on the basis of litter size over twice as high as either treated-male column and about four times the control record. The proportion of late prenatal to postnatal mortality is in the first female column over three to one and in the last column over six to one. There were some defective animals in both female groups. The records of the females in this and the two preceding tables are out of accord with the records from fowls and do not fit an explanation based on a germinal selection or partial infertility. The total productivity is good and the late prenatal and the postnatal mortality are high.

It is seen at once that the records from the treated-female generations are far worse than from the treated-male generations; in fact, so much worse that we are led to conclude that the alcohol has not acted on exactly the same things in the two cases. The increased effect of the treatment in the double female column is much more evident than in the two male generation column. The results in the male columns are due only to an action of the treatment on the spermatozoa or male germ cells, while the results in the female columns are also due to the effects of the treatment on the germ cells or ova, but more largely to the effects of the alcohol on the developing embryos within the uterus of the treated mother. Provided the effects of alcohol were equal on the sperm and ova of guinea-pigs, the difference between these two sets of records would then represent the action of the treatment on the developing embryo itself.

Although the records in table 5 involve only small numbers, we are led to believe that they represent the true trend of the effects, since they harmonize so perfectly with the data of different composition yet much more complete shown in tables 3 and 4.

Here again, as in tables 3 and 4, the treated-male lines show the early prenatal mortality (based on the average litter size and frequent mating failures) to be unusually high while in the female line it is low. In the male lines the late prenatal and

total mortality is low while in the female lines the late pre-
natal mortality is extremely high and the total mortality very
great.

Finally, this table may be considered as supplying evidence of
the increased effect of higher or longer alcoholic dosage. The
double male records which have usually been derived from ani-
mals that have had longer or more treatment during the two
generations are somewhat inferior to the one generation male
treated records, and this inferiority is very much more decided
for the female groups in the case of the higher-dosed two-genera-
tion records.

11. THE SEX-RATIO IN RELATION TO PATERNAL AND MATERNAL ALCOHOLISM AND TO THE TREATMENT OF MALE AND FEMALE ANCESTORS WITH ALCOHOL

In the last group of table 3 it will be remembered that all of
the non-inbred acoholic descendants were separated into three
classes with only father alcoholic, only mother alcoholic, and
both parents alcoholic. Again, in the last group of table 4,
these 594 animals were rearranged into three classes, from only
male ancestors treated, only female ancestors treated, and both
male and female ancestors treated. The difference between
these classifications are made clear in the discussion of tables 3
and 4. If we now record the number of males and females com-
posing each of these six classes and express their sex-ratios on
the basis of the number of males to every 100 females, a most
peculiar result is obtained, and one for which it is very difficult
to give a completely satisfactory explanation.

The number of males and females and their mortality records
in each of the six classes are shown in table 6. As a standard of
comparison the 233 control animals are similarly recorded in
this table. For further comparisons a total sex-ratio and the
sex-ratios for animals born in different size litters are given be-
low the table. The total sex-ratio calculated for about 1600
animals is 109.6; that is, 109.6 males to every 100 females. Many
of these animals were from alcoholic lines, so that this sex-ratio
may not be exactly normal. Yet a further perusal of the table

TABLE VI

THE SEX-RATIOS OF THE PROGENY AFTER THE TREATMENT OF MALE AND FEMALE ANCESTORS OR FROM ALCOHORIC FATHERS AND MOTHERS

	Normal line			Only father alcoholic			Only mother alcoholic			Both parents alcoholic			Only ♂ ancestors treated			Only ♀ ancestors treated			♂ and ♀ ancestors treated		
	♂	♀	Unknown sex	♂	♀	Unknown sex	♂	♀	Unknown sex	♂	♀	Unknown sex	♂	♀	Unknown sex	♂	♀	Unknown sex	♂	♀	Unknown sex
Total number	120	106	7	59	58	4	121	125	28	103	85	11	107	98	18	77	89	15	99	81	10
Sex ratio	113.20 (2.77)			101.72 (2.41)			96.80 (2.69)			121.17 (2.28)			109.18 (2.42)			86.51 (2.66)			123.58 (2.37)		
Lived over 3 months	97	84	0	41	38	0	85	81	1	73	64	0	72	68	1	51	57	0	76	58	0
Total dead	23 19.1%	22 20.75%	7 100%	18 30.5%	20 34.48%	4 100%	36 29.75%	44 35.20%	27 96.42%	30 29.12%	21 24.70%	11 100%	35 32.7%	30 33.67%	17 94.44%	26 33.76%	32 35.95%	15 100%	23 23.25%	23 28.39%	10 100%
Percentage of total dead	19.91%			32.47%			32.52%			27.12%			31.10%			34.93%			25.55%		

Total mortality of ♂ 29.68%
                  "   ♀ 31.71%

Total sex ratio 109.63
" " of 1 in litter animals   113.18 (194)
" " of 2 in " "   114.86 (578)
" " of 3 " "   101.74 (579)
" " of 4 " "   106.66 (248)

will suggest a tendency on the part of the different alcoholic lines to level the sex-ratio to normal when they are combined as a grand total, yet we are not by any means comparing the sex-ratios from the alcoholic lines with an average alcoholic ratio. The sex-ratios of 194 animals born one in a litter was 113; of 578 born two in a litter, sex-ratio 114.8; of 579 born three in a litter, sex-ratio 101.7, and of 248 animals born in litters of four, the sex-ratio was 106.6.

The first column of table 6 shows that of the 233 non-inbred control animals, 120 were males and 106 were females. The proportion of males to females is thus 113.2 to 100; that is, a sex-ratio of 113.2. The average-size litter in which these animals were born is shown in parentheses in the sex-ratio space as 2.77. The mortality record for the males was about the same as for the females, having only a very slight advantage.

The third column of animals from only mother alcoholic are also in the majority of cases individuals from only female ancestors treated, the sixth column, but not entirely so, as many of the mothers may have been alcoholic on account of a treated father or grandfather. In general, however, the third and sixth columns are rather the same in composition, the sixth being a purely female treated group while the third column is largely so but not entirely, and while not necessarily to be treated together they may be considered in connection with one another. A point of immediate notice is that the sex-ratios in both of these columns, 96.8 and 86.5, are very low.

When only the father is alcoholic, second column, or only the male ancestors are treated, fifth column, the sex-ratios are higher, 101.7 and 109.1. While if both parents are alcoholic, fourth column, or male and female ancestors are treated, seventh column, the sex-ratios are very high, 121.1 and 123.5. It must also be noticed that these differences in sex-ratios are more accentuated in the last three columns, giving the descendants from only female ancestors treated with alcohol the lowest sex-ratio in the entire table; those from only male ancestors treated a considerably higher ratio, and from both male and female ancestors treated the highest sex-ratio for all groups. A differ-

ence of 37 between the number of males to 100 females in animals from treated-female ancestors as compared with those from both male and female ancestors treated is indeed very great.

Are these differences in sex ratio a result of the direct influence of alcoholism upon sex determination or sex differentiation? Or are they indirectly brought about by a difference between the early prenatal mortality rates of the two sexes in the several groups considered? Or are these merely chance differences? There is no doubt that chance plays a large part in the make up of all sex-ratios, but to be consistent in six straight cases as the six groups show can scarcely be dismissed as a chance result.

It is very peculiar that these different sex-ratios should coincide in a direct manner with differences in the early prenatal mortalities among the several groups of table 6, and thus suggests that the explanation for the sex-ratio differences may be in part at least along the line of the second of the above propositions. After considering this probability of differences between the early prenatal mortalities of males and females, we may then discuss the further possibility of the direct effects of the treatment on the sex-ratios.

The groups having the lowest sex-ratios, female lines with ratios 96.8 and 86.5, also have, as shown in tables 3 and 4, the largest average litters, 2.69 and 2.66, or the lowest early prenatal mortality. The lines having a somewhat higher sex-ratio, male lines with ratios 101.7 and 109.1, have correspondingly somewhat higher early prenatal mortalities, as indicated by the smaller average litters, 2.41 and 2.42; while the lines having the highest sex-ratios, double lines with ratios 121.1 and 123.5, have along with these the highest early prenatal mortalities as shown by the smallest average-size litters, 2.28 and 2.37.

This is certainly a very suggestive parallelism. And if one now considers the fourth line of the table giving the total dead of each sex, in every column, with one exception, it will be seen that the female mortality is higher than the male. The exception is in the column from both parents alcoholic; here the

sex-ratio is very high and yet the late male mortality is higher than that for the females.   The total mortality for the females is higher than that of the males, 31.71 per cent against 29.68 per cent.   It is further shown below the table that small litters have a higher sex-ratio than large litters, the sex-ratios for litters of one and two young being respectively 113.1 and 114.8.   While for litters of three and four young the sex ratios are 101.7 and 106.6.   It has been pointed out before that the small litters are often due to an early prenatal mortality which has destroyed some of the original members, and since the sex-ratios of such litters are high the majority of embryos dying may have been females.   We may see finally by a study of table 7 that female animals are generally smaller at birth than males in the same litter, and as their total higher mortality would indicate, they are probably also weaker.

TABLE VII
THE BIRTH WEIGHTS OF MALE AND FEMALE MEMBERS OF MIXED LITTERS

	Number of Litters	Total weight of males in grams	Total weight of females in grams	Total excess Weight of males over females	Average excess weight of males over females	Percent of excess weight of males over females
Litters of 1♂ 1♀	105	7861	7525	336	3.20	2.18%
Litters of 2♂ 1♀	136	9513	4654 (9308)	205	0.75	1.08%
Litters of 1♂ 2♀	125	4428 (8856)	8790	66	0.26	0.37%
Litters of 2♂ 2♀	36	2325	2239.5	85.5	1.19	1.87%

Table 7 only includes mixed litters; that is, those containing both male and female members.   It shows that in 105 litters of two animals of opposite sex the total birth weight of the 105 males was 7861 grams and of the 105 females only 7525 grams, or 336 grams less.   The average excess weight of males over females in these litters of two was 3.2 grams, giving a percentage of excess weight of 2.18 in favor of the males.

One hundred and thirty-six litters of three, consisting of two males and one female, are recorded.   The total weight of the

272 males was 9513 grams; that for the 136 females was 4654 grams.   If this total weight be doubled for comparison with the total weight of the double number of males, we have 9308 grams.   The males again have a total advantage, amounting here to 205 grams.   The average excess weight of the males is 0.75 gram, or a 1.08 per cent excess weight of males over females.

It will be noted in this table that the average weight of the individuals is very low.   This is due to the fact that a number of abortions in which the sex could be distinguished, as well as premature still-births are included.   These small specimens have brought the average in some cases almost below the birth weight which permits survival.   It is also noticed that the 272 males in the second line only weigh about one-quarter more than the 105 males in the first line of the table, and this is due to the fact that there were many more abortions and early premature births of litters consisting of three individuals than of two.   While the males in the litters of one male and one female averaged almost 75 grams, the males in litters of two males and one female averaged less than 35 grams.

The third line of the table shows 125 litters of one male and two females.   The 125 males weighed 4428 grams which may be doubled to give 8856 grams for comparison with the total weight of 8790 for the 250 females.   There is a total advantage of 66 grams in favor of the males.   The average excess of male weight is 0.26 gram, or 0.37 per cent over female weight.

The last case of thirty-six litters, consisting of two males and two females each, gives a total excess of 85.5 grams to the males. The average excess weight of the males over females is 1.19 grams, and the per cent of excess of males over females is 1.87.

It is thus seen that the males born in litters consisting of both sexes possess a superiority in body weight over the females in every combination.   We do not attribute this constant excess in favor of the males to a sexual dimorphism in size.   In a group of guinea-pigs both young and adult females are often larger in size than comparable males, and no constant size difference between the two sexes is known.   It seems more probable that

this advantage in weight on the part of the males, the majority of which are of alcoholic ancestry, is in line with the lower mortality records of the males shown in the various columns of table 6. And this may further bear on the explanation of the high sex-ratios in those lines with high early mortalities or small average litters.

There is, therefore, much evidence to indicate that among alcoholic guinea-pigs the females very probably suffer a much higher early prenatal mortality than do the males, and it is shown that the female mortality is higher than that of the males at all other periods, table 6.

Before proceeding further with our theoretical explanation of the different sex-ratios in the several groups, which leads finally to a consideration of views expressed in a previous communication, still another important relationship may be pointed out between early prenatal mortality and the sex-ratio, on one hand, and the late prenatal and postnatal mortality, combined in table 6 under 'total dead,' on the other. Stated concisely, the higher the sex-ratio and the early prenatal mortality, indicated by the small average litter, the lower will be the total late prenatal and postnatal mortality, and vice versa. The columns with the highest sex-ratios, 123.58 and 121.17, and at the same time the highest early prenatal mortalities or the smallest average litters, 2.37 and 2.28, show the lowest late mortalities, 25.55 and 27.12 per cent. In the opposite way the columns with the lowest sex-ratios, 96.8 and 86.51, and the lowest early prenatal mortalities, or the largest average litters, 2.69 and 2.66, have the highest later mortalities, 34.93 and 32.52 per cent. This is in line with what was brought out during the discussion of table 4 showing that the higher the early prenatal mortality, or the smaller the average litter, the lower will be the late prenatal and postnatal mortalities.

There is one very evident objection to the foregoing explanation of the peculiar sex-ratios as being due to a differential sex mortality during the early prenatal periods. That is, among the normal stock the sex-ratio is rather high, although the early prenatal mortality is probably very low as indicated by the

large average litter. With a large average litter the sex-ratio should be very low as in the female lines. We could only avoid this difficulty by assuming that the control lines are out of the consideration, since the other sex-ratios being discussed are all shown by modified alcoholic groups among which entirely different conditions obtain from those existing in the control. Whereas there are reasons for such a position, it would seem preferable at present to admit that the case of the control is a real objection. And such an objection would serve to indicate that while a higher mortality on the part of the female embryos in the alcoholic groups might actually exist, yet it accounts only in part for the peculiar sex-ratios found. A recognition of the normal record also makes it difficult to account for the very low sex-ratios of the female lines. Here the early prenatal mortality was low on the basis of the average size litter, but if any early prenatal mortality did occur it could not have been partial to the female embryos, but must on the contrary have been confined almost totally to male embryos or else a sex-ratio could never fall 25 below the control. Is it possible that wherever a treated male is concerned, as in the male columns and the double columns of table 6, there is a high early prenatal mortality among the female embryos, and on the other hand where only a treated female is concerned there is a high early male mortality? It is difficult to believe so, and therefore differences between the early mortalities of the sexes can, on our present data, only partially explain the sex-ratios found in table 6. This leads to a final explanation which may seem highly theoretical, yet it does have a basis of fact.

In an earlier communication (Stockard and Papanicolaou, '16), we presented some evidence which seemed to indicate a possibility that the action of the alcohol treatment not only differed in its effects upon the two sexes treated, but also acted differently on the two groups of spermatozoa in the male, the so-called male-producing and female-producing sperm.

We suggested that the action of the treatment was more severe on the germ cells of the male than on those of the female; in other words, that the spermatozoa were more susceptible

than the ova.   The inferiority of the column from male ances-
tors treated as compared with that from female ancestors treated
in the second group of table 4 seems to substantiate such a
position.   The possibility exists, however, that the treatments
of the male and female ancestors may not have been equally
severe, since they have been treated in different fume tanks.
This question is now being studied.   At any rate, we believe it
is proved that the germ cells of the female are as definitely in-
jured and modified by the treatment as are the germ cells of the
male.   This is the point of importance in the present connection.

The female offspring from treated fathers were found in the
report cited to be inferior as a group to the male offspring as
regards their powers of existence and structural perfection.   The
opposite was indicated among the offspring of treated mothers,
the males being inferior to the females.   Our explanation of
these conditions was that the two classes of spermatozoa which
differ structurally also differ in the degrees of injury suffered
from the treatment.   We are further testing these suppositions
by selected matings and hope to report on them in the future.
For further details regarding the supposed differences between
the behavior of the two classes of spermatozoa, the reader is
referred to our 1916 paper.

An explanation of the sex-ratios in table 6 may now be given
along similar lines and the peculiarities found among these sex-
ratios are exactly in accord with our previous theoretical consid-
erations.   If the male guinea-pig does possess, as has been claimed
(Stevens, '11), heteromorphic spermatozoa, one class with a
small Y chromosome, the male producing, and the other class
with a larger X chromosome, the female producing, the follow-
ing may be assumed: In the treated-male lines the female-pro-
ducing spermatozoa are more decidedly affected, possibly on
account of their larger quantity of chromatin, and therefore, in
the competition to fertilize the eggs they are not so successful
as the less injured male-producing sperms.   Consequently, more
male animals are produced than female.   Or, if the female-
producing sperm are not in any or all cases actually prevented
from fertilizing eggs, nevertheless the individuals produced by

such a fertilization are inferior and more apt to die during early developmental stages, and thus a greater number of male embryos would survive and be born.

When the alcoholic mother or early female treated lines were mated with untreated normal males, the sons were inferior to the daughters. Here again, taking into consideration the two structurally different classes of spermatozoa, the normal males paired with alcoholic females contribute a smaller amount of normal chromatin to the complex producing male offspring than to that giving rise to the female offspring. The records of the males are hence inferior to those of the females. And in the present connection such males might be expected to suffer a higher early prenatal mortality and so give rise to the very low sex-ratios shown by the columns from 'only mother alcoholic' and 'only female ancestors treated.'

Such reasoning from the present data is admitted to be highly speculative; nevertheless, if the morphological differences which have been found to exist between the two classes of spermatozoa in a number of animal species have any significance, they must sooner or later be recognized as the underlying cause of such results as table 6 shows for sex-ratios in alcoholized guinea-pigs.

These ideas also account for the fact that the sex-ratios of the normal animals is out of accord with the ratios of all the treated groups on the basis of the average litter size. This discord was recognized as a possible objection to the purely differential sex mortality explanation previously discussed. In the present connection we may take the following position.

The normal group has been subjected to no injurious action which has tended to modify the expression of the sex-ratio, while in the alcoholized groups there is evidence of a deviation from the normal, in one direction or the other, depending upon the combination concerned. And this deviation is imagined to be due to a lower fertilizing ability on the part of certain spermatozoa.

There is another question to be considered in connection with the differences in response on the part of the two classes of spermatozoa; that is, the possibility of certain eggs being more sub-

ject to fertilization by either the X or Y type of spermatozoa. Even though the egg might be practically equally accessible to both types under normal conditions, a peculiarly affected egg might become much more readily fertilized by one class of sperm than the other, and almost all male offspring might result in one case and females in the second. One might feel that these are large suppositions on the basis of the minute differences between the two groups of sperm. But it may be replied that the differences are only minute from the standpoint of the minuteness of the structure considered. Corresponding differences between great things would necessarily seem much more important, but with present powers of observation only very great differences between cellular structures are visible at all.

There is evidence from a study of the control of sex-ratios in normal guinea-pigs to indicate that certain females have a very strong tendency to produce male offspring regardless of the male with which she is paired (Papanicolaou, '15). Other females have as decidedly marked tendency to produce female offspring. Such females may be said to have either a male or female tendency, while other females are in this regard indifferent, producing as many offspring of one sex as of the other. These tendencies may be explained in accord with the above discussion as due to a high affinity for one type of sperm on the part of the ova of one female, while the ova of another female are particularly susceptible to fertilization by the other class of sperm. The indifferent females are those with ova which are fertilized equally as well by one type of spermatozoa as the other. There are striking cases among the ascidians and other forms illustrating selective fertilization, and the above suggestions are by no means without foundation.

Certain male guinea-pigs are also known to have a strong tendency to beget female offspring regardless of the females with which they are paired. Other males have a high male-producing tendency and still others are more or less indifferent in their sex-determining quality. This may be readily imagined to result from a difference in the activity or fertilizing powers of the

two types of spermatozoa in certain male animals. And there is evidence to show, as cited in our previous paper, that the fertilizing power of the spermatozoa may be modified in such a way as to render them much less capable of success. If this is the case, we may be justified in assuming that one class of sperm may often, even under normal conditions, be at a disadvantage as compared with the other. It is even more probable that under modified conditions the two morphologically different classes of spermatozoa will not be affected to equal degrees.

In conclusion, then, it seems highly probable that the peculiar sex-ratios shown by the several groups of treated animals recorded in table 6 are in part due to differential sex mortalities during early prenatal stages, on account of the close correlation between the sex-ratios and the average litter sizes. This difference in early prenatal mortality between the sexes does not, however, completely satisfy the case. The sex-tendency of the animals considered and the possibility in the case of delicate treatment of affecting the two types of spermatozoa in different ways or degrees are certainly factors to be recognized in the production of the results obtained.

Pearl found that for fowls treated with alcohol the relative proportions of the sexes produced were not significantly different from normal control series. Our results for the sex-ratio of the total alcoholic series agree with Pearl's findings. The sex-ratio of the 594 alcoholic animals considered in the present paper is 105.6, which, in view of the numbers involved, is not significantly different from the control series. Yet studying separately the several groups shown in table 6, we find strikingly wide differences in the sex-ratios and the arrangement of these differences is decidedly consistent. From the standpoint of the above discussion it seems to us legitimate to consider the six groups individually, or at least as three classes, since there is a probability that different processes or conditions are affecting the results in the different cases. Several recent experiments on the modification of the sex-ratio would tend to strengthen such a probability.

12. THE BIRTH WEIGHTS AND RATE OF GROWTH IN THE NORMAL AND THE ALCOHOLIC SERIES

In the present section the birth weights and ability to grow of the animals born in the normal and the alcoholic series may be compared. Here again comparisons must be made between animals born in litters of the same size. It may be expressed generally, as was done above for the mortality rate, that the birth weight of an animal, either normal or alcoholic, varies inversely with the size of the litter in which it is born. The average daily increase in weight during the first month varies in the same way. So that when one month old the weight of a guinea-pig also as a rule varies inversely with the size of the litter in which it was born. This condition holds up to three months, at which time the guinea-pig is mature. But the daily gain in weight during the second and third months after birth ceases to be greatest for the members of small litters. Yet the advantage in growth rate comes to the members of the large litters at so late a time that they are unable to make up their disadvantage sufficiently to equal in size the members of small litters within three months. All of these statements apply equally to both the alcoholic and normal series, and thus the influence of the litter size in general is the same in both cases.

The question then arises whether there is an actual difference in birth weights and growth rates between the two series. Table 8 contains the birth weights of 225 normal control and 531 animals of the alcoholic series. This alcoholic group, as the foregoing tables show, not only includes F_1 animals, or offspring from directly treated parents, but also their descendants for several generations, F_2, F_3, and F_4. The animals of both series are arranged in table 8 according to the size litters in which they occur.

A review of the table shows that the normal series is superior in the average birth weight of the individual and the average birth weight of the entire litter, as well as the average birth weight of the individual born in each of the five different-size litters.

TABLE VIII
BIRTH WEIGHTS AND RATES OF GROWTH OF NORMAL AND ALCOHOLIC YOUNG

	Normal					Alcoholic				
Weight at birth	1 $\frac{1085}{10}$ (108.5)	2 $\frac{3779}{46}$ (82.15)	3 $\frac{6367}{87}$ (70.88)	4 $\frac{4125}{67}$ (61.56)	5 $\frac{941}{15}$ (62.73)	1 $\frac{4028}{41}$ (98.42)	2 $\frac{13867}{168}$ (82.54)	3 $\frac{14741}{225}$ (65.51)	4 $\frac{5200}{92}$ (56.52)	5 $\frac{244}{5}$ (48.80)
	(Average 77.16) Average productivity 197.12 225					(Average 70.35) − 9.23% (of the mean) Average productivity 170.0 531				
Weight at the end of the first month	1 $\frac{2228}{7}$ (318.28)	2 $\frac{8902}{31}$ (240.54)	3 $\frac{14288}{68}$ (210.11)	4 $\frac{6750}{37}$ (182.43)	5 $\frac{2302}{12}$ (191.83)	1 $\frac{8633}{29}$ (297.68)	2 $\frac{30461}{131}$ (232.52)	3 $\frac{26398}{133}$ (198.48)	4 $\frac{6881}{40}$ (172.02)	5 $\frac{338}{2}$ (169.0)
	(Average 228.64)					(Average 213.94) − 6.63%				
Average daily increase in weight during the first month	6.99	5.28	4.64	4.02	4.30	6.64	4.99	4.43	3.85	4.00
	(Average 5.04)					(Average 4.78)				
Weight at the end of the third month	1 $\frac{4013}{8}$ (501.62)	2 $\frac{16454}{38}$ (433.0)	3 $\frac{25643}{62}$ (413.54)	4 $\frac{12552}{33}$ (380.36)	5 $\frac{4764}{12}$ (397.0)	1 $\frac{14264}{31}$ (460.12)	2 $\frac{44635}{105}$ (425.09)	3 $\frac{41751}{102}$ (409.32)	4 $\frac{11749}{32}$ (367.15)	5 $\frac{718}{2}$ (359.0)
	(Average 425.11)					(Average 404.13) − 5.06%				
Average daily increase in weight during the 2nd and 3rd months	3.05	3.20	3.39	3.29	3.41	2.70	3.20	3.51	3.25	3.16
	(Average 3.26)					(Average 3.16)				

The average birth weight of the individual in the normal series is 77.16 grams against 70.35 grams for the alcoholic, and the average litter weight is 27.12 grams heavier among the normal animals. The average weight of the individual in a given size litter is shown in parentheses below the litter number; this is obtained by dividing the total weight in grams of all such litters by the total number of animals composing them. For example, in the alcoholic series there are 168 animals born in litters of two and their total birth weight was 13,867 grams, which gives an average weight of 82.54 grams per individual. The average weight of the individual is lower in the large litters than in the small ones in both series.

The second line of the table shows in a similar way the total weight at the end of the first month of all individuals in the several-size litters and below this the number of individuals concerned in each case. The quotient obtained by dividing the total weight by the number of animals is given in parentheses as the average weight of the individual in each litter at one month old. At this age the average weight of normal animals

in litters of one was 318.28 grams against 297.68 grams for the alcoholic litters of one.   The general average weight at one month for the normal series was 228.64 grams against a general average of 213.94 grams for the alcoholics.

The average daily increase in weight during the first month is given in the third line of the table.   It shows a mean daily increase for normal animals of 5.04 grams and for alcoholic animals only 4.78 grams.   Members of small litters in both groups gained more rapidly than members of large litters.

The weights at the end of the third month, when the animals are about mature, are given in the fourth line of the table.   Normal animals born one in a litter average over 500 grams, while comparable alcoholic animals weigh only 460.12 grams.   The average normal animal at three months old weighs 425.11 grams against an average of 404.15 for the alcoholic animal.

The last line shows that the average daily gain in weight during the second and third months was about as great for the alcoholic animals as for the normals.   A much greater selection or elimination has taken place previous to this time among the alcoholic series than among the normal, as a reference to any of the mortality tables will show.

All in all, table 8 would seem to indicate that in every case the normal offspring weigh more and grow more rapidly shortly after birth than do the young alcoholic specimens.

The several points considered above and their general meaning may be much more clearly expressed in the diagram, figure 9. On the left side of the diagram are shown the records for the alcoholic series and the normal records are on the right.   The shaded right-angle triangles represent the difference in average weight between the individuals in litters of one, two, three, four, and five at birth, at one month old, and at three months old from the two series.   The altitudes of the right triangles measure the magnitude of the differences.

Animals born one in a litter in the alcoholic and the normal series, as the bottom short triangle indicates, show a greater difference in weight than those from any other size litter except that consisting of five individuals as the low long triangle repre-
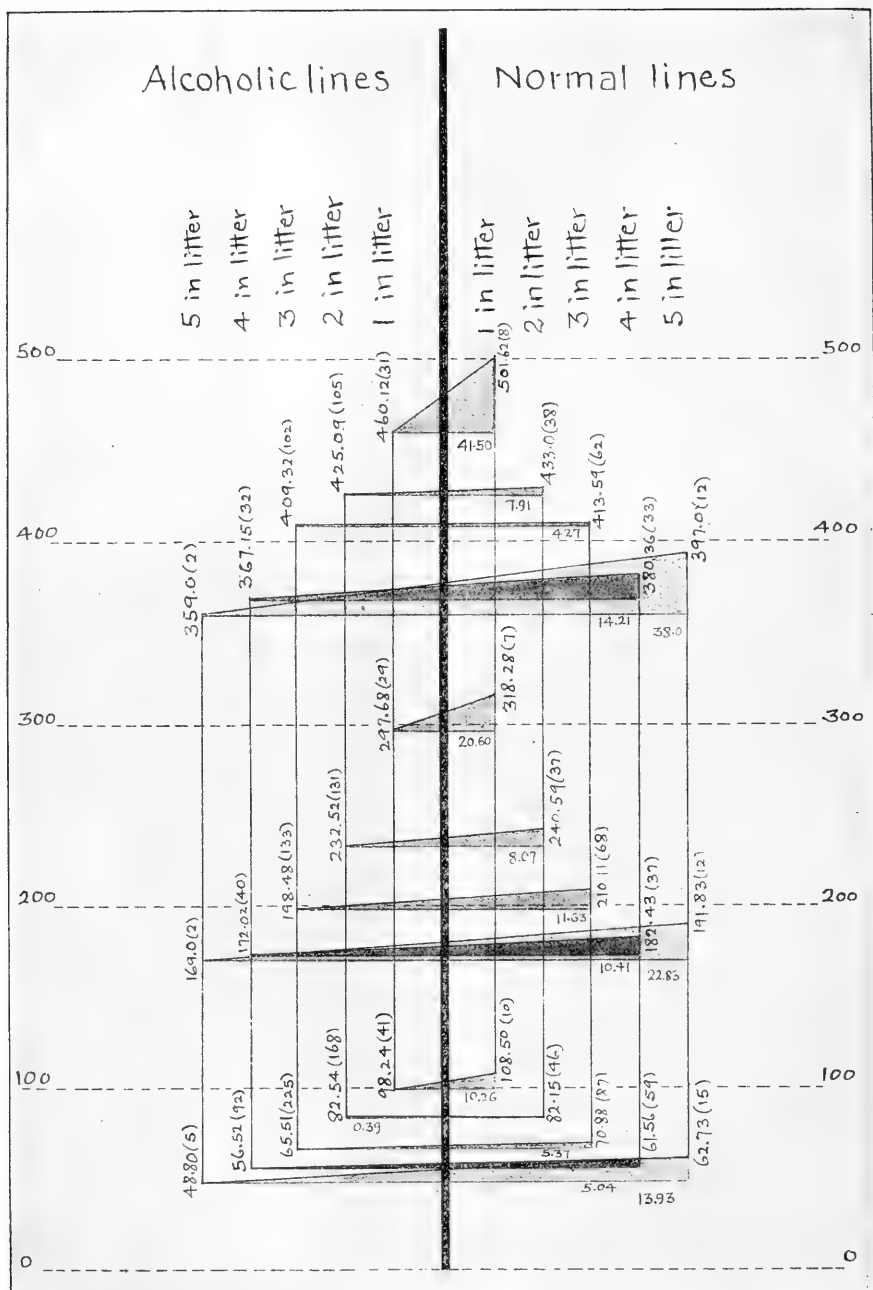
Fig. 9 Diagram illustrating the differences in weight between normal and alcoholic line animals born in litters of the same size. The weights are given at birth, at one month, and at three months old. Further explanations are to be found in the text.

sents. There is little difference between the birth weights of normal and alcoholic animals born in litters of two, three or four.

When one month old the middle group of triangles representing by their position the weights in grams again show the largest differences between alcoholic and normal animals in litters of one, the short triangle, and litters of five, the long triangle. The normal animals in litters of one have passed the 300-gram line in weight, while the average alcoholic member of a litter of five weighs only 169 grams. Members of the two series in litters of two, three, or four do not show very great weight differences.

The top triangle shows a very large difference in weight at three months between normal and alcoholic animals born one in a litter. The triangles for two and three in a litter animals are almost flat at three months, indicating very little difference between such normal and alcoholic animals. Alcoholic members of litters of four are somewhat smaller in average than normal, while alcoholic from litters of five are far below the normal in weight as the long triangle shows at three months.

We have here an example of the influence of the alcohol effect combined with the action of a normal condition, the condition being the size of the litter in which the animal is born. From a consideration of the diagram we may, therefore, conclude, first, that normal-stock animals born one in a litter are so strong as to run far ahead of the one in a litter alcoholic animals, although the latter at birth, at one month, and at three months are much heavier than all normal animals born in larger litters at similar periods. Consequently, the advantage of developing alone in the uterus is sufficient, so far as birth weight and rate of growth are concerned, to overcome the disadvantages resulting from alcoholic ancestry to such a degree that these individuals are better than control animals developing in larger litters. Yet in birth weight and growth rate these singly born alcoholic animals are further behind the singly born control than are the alcoholics from any other size litters behind the control from the same size litters. Thus, although being born alone tends to overshadow the alcohol effect, nevertheless the effect is still shown by comparison with control specimens born alone.

If we now recall the fact that alcoholic animals produce more small-size litters than do the control, and recognize that members of small litters in all cases weigh more, grow faster, and are more apt to survive than members of larger litters, it becomes evident that the production of a high percentage of small litters is a fortunate provision tending to preserve the alcoholic stock by counterbalancing to some degree the magnitude of the effects induced by the alcoholism.

Second, animals born in litters of two or three have a tendency to weigh the same at birth and to grow at a similar rate during the first three months, whether they are from the normal or alcoholic stock. In other words, being born in litters of this size gives no great advantage to the normal animals over the alcoholics, as does being born in litters of only one. Or stated reversely, members of litters of two or three are not placed at a great disadvantage so far as birth weight and growth rate are concerned on account of their alcoholic ancestry, as is found below to be the case for the members of larger litters.

In the third place, when animals are born in litters of four the alcoholic stock are at a disadvantage in birth weight when compared with the normal. The rate of growth of the alcoholic animals from litters of four is also slower than that of the comparable control animals.

Lastly, in the fourth place, alcoholic animals born five in a litter are very small and weak and only a few survive, yet these selected few fall far behind the normal animals from litters of five in their rate of growth. Thus at three months there is a greater difference in average weight between the alcoholic and control members of litters of five than between the members of any other size litters in the two series, except the animals born singly. The alcoholic animals as a group are at a disadvantage in birth weight and rate of growth, but when born in large litters of four or particularly five, this disadvantage is greatly exaggerated by the handicap which befalls the members of all large litters, the control as well as the alcoholic.

13. THE RECORDS OF NORMAL MALES AND FEMALES PAIRED SUCCESSIVELY WITH NORMAL AND ALCOHOLIC MATES: THE CRUCIAL DEMONSTRATION OF THE EFFECTS OF ALCOHOLISM ON THE OFFSPRING

When the records of any group of experimental animals are compared with the records of a normal group, the possibility presents itself that some selection either conscious or unconscious may have played a part in forming the groups. Such a source of error is no doubt practically eliminated by many well-known methods of choosing control and experimental animals from a given population. We believe such a defect is entirely insignificant in the foregoing records which have involved many animals through several generations from the same stocks in the case of both the experimented and the control. It is, nevertheless, satisfactory to consider the records of the same normal animals paired successively with control animals and with animals of the alcoholic lines. Table 9 presents all of the mating records of fourteen normal males and fifteen normal females that have been paired in this way. This table gives a most perfect control and shows most clearly the alcohol effects.

TABLE IX

NORMAL MALES AND FEMALES PAIRED SUCCESSIVELY WITH NORMAL AND ALCOHOLIC MATES

	Individual matings of 14 normal males, each one mated successively with		Individual matings of 15 normal females, each one mated successively with	
	Normal females	Alcoholic females	Normal males	Alcoholic males
Number of matings	36	44	26	23
Total number of young	86	100	59	50
Negative result	2 (5.55%)	4 (9.09%)	1 (3.84%)	5 (21.73%)
Lived over 3 months	65	58	51	30
Total dead or died within 3 months	21 (24.41%)	42 (42.0%)	8 (13.55%)	20 (40.0%)
Defective	0	6 (6.0%)	0	5 (10.0%)

The fourteen male animals are in no sense selected; they are all of the normal males in our series of animals between the numbers 613 and 1909 which have been mated with both normal and alcoholic females. The record numbers of these males are 665, 666, 667, 669, 670, 676, 677, 679, 681, 682, 683, 854, 914, and 1052. The fourteen males, as the table shows, have been mated in all eighty times. The fifteen females recorded include also every normal female among the animals considered in this paper that has been paired with both normal and alcoholic males. The record numbers of the females are 645, 646, 650, 652, 657, 661, 662, 671, 674, 675, 703, 722, 760, 890, and 1043. These have been mated in all forty-nine times.

There has been no selection or choice in mating these animals or in estimating the results, since it was only decided to arrange such a table after beginning the present study of the data.

The first column of table 9 shows the results of thirty-six matings of the normal males with normal females. Two of the thirty-six matings failed to produce results, or 5.55 per cent, and the remaining thirty-four matings gave rise to eighty-six young. Sixty-five, or 75.59 per cent, of these lived to reach maturity, while 24.41 per cent died within three months. None of the eighty-six offspring showed any gross structural defects.

When these same normal males were mated forty-four times with alcoholic females, the second column shows that four matings failed, or 9.09 per cent, almost twice as many as the failures with normal females. The forty successful matings produced one hundred offspring, only fifty-eight of which were capable of survival to maturity. Thus 42 per cent of the young animals died within three months against only 24.41 per cent of those from the normal mothers and same fathers. Six per cent of the young from the alcoholic mothers possessed noticeable structural defects.

In every respect the matings of the fourteen normal males produced greatly superior results when paired with normal females, as compared with their records by alcoholic females. The numbers are comparatively small, but the differences are large and the inferior records are consistently in the same column.

The third and fourth columns contain similar records from the matings of the fifteen normal females with normal males and with alcoholic males.   The twenty-six normal matings gave only one failure, while the twenty-three matings with alcoholic males failed to give results in five cases, or in 21.73 per cent of the trials. The alcoholic males always give a high percentage of mating failures even with normal females and, as this case shows, with females giving only a low per cent of failure by normal males.

The normal matings produced fifty-nine young, fifty-one of which survived while only eight, or 13.55 per cent, died within three months.   This is an unusually low mortality record and proves the ability of these females to produce strong viable young. None of the offspring from the normal matings were defective.

The same females produced by alcoholic males fifty young, only thirty of which lived to maturity.   Therefore, 40 per cent of them were non-viable, which is three times more than was the case with offspring from these females by normal fathers. Ten per cent of the fifty offspring were defective.   The contrast between the two groups of results from the same females is so great that the possibility of the difference being due to the smallness of the numbers involved would seem to be completely eliminated.   The records in the entire table are perfectly consistent and very clear cut.

It would seem only proper to interpret such results, along with the mass of evidence in the foregoing pages, as showing that alcoholic guinea-pigs, whether directly treated or descended from treated individuals, have had their ability to produce strong, viable offspring definitely and decidedly lowered.   And it may be added in this connection that evidence from purely male treated lines as well as that given by later generations from the female treated and mixed lines, points directly to the fact that the germ cells have been affected.   The effects of this modification are transmitted through several generations, only to be lessened by the elimination through death and sterility of the weakest individuals from the mating records and the constant introduction of more and more normal germ plasm into the line by matings with the normal stock.

14. THE CONTRASTED QUALITIES IN THE CONTROL AND THE ALCOHOLIC SERIES

The earlier reports on these experiments have given in the general text the various differences between the alcoholic and control lines; the case is made much clearer, however, if all the contrasted qualities be arranged together in summary fashion. In Pearl's recent report on the influence of alcohol inhalation on the progeny of the domestic fowl, he has given a concise arrangement of the differences between the records of the experimented and control lines. The several qualities he has compared such as mortality records, fertility, abnormalities, etc., are the same as those considered in our previous papers. We have here constructed a similar table to the one used by Pearl to show the qualities contrasted in the former sections of this paper. Definite numerical values have been presented for fourteen different qualities studied in the two groups of animals. Several of these qualities are closely related, such as weights after different periods of growth and the mortalities calculated at different periods, yet these are stated separately since they were measured in this manner and help somewhat to give a clearer analysis of the entire problem.

Table 10 shows the qualities measured. The first column of figures are the records from the control, the second column are the alcoholic records. In the last column a − sign indicates that the alcoholics are inferior to the control for the given quality; a zero, that the two groups are similar in the given respect, and a + sign would show that the alcoholics are superior to the control. It is seen at once that the alcoholic series suffers by comparison in every case except one, and in this case the two series are equal on account of an earlier unusually large difference.

The alcoholic guinea-pigs are less productive, giving litters of smaller size than the normal, their matings more often result in failure to conceive; associated with these two facts there is a higher early prenatal mortality which is the only quality included in the table that cannot be numerically expressed for reasons brought out in previous pages.

TABLE X.
QUALITIES CONTRASTED BETWEEN THE NORMAL AND ALCOHOLIC PROGENIES

Qualities measured	Normal	Alcoholic	Alc. Sup.+ Alc. Inj.−
1. Size of litter	2.77	2.47	—
2. Failure to conceive	4.45%	13.04%	—
3. Early prenatal death (size of litter, failure, etc.)	low	high	—
4. Proportion late prenatal death	51.92%	70.14%	—
5. Post-natal mortality	10.70%	10.60%	O
6. Total mortality	22.31% (100)	35.52% (189)	—
7. Abnormalities	O	2.52%	—
8. Oversize (+500 grs. at 3 mos.)	5.57%	2.86%	—
9. Undersize (−300 grs. at 3 mos.)	0.42%	1.34%	—
10. Late generations alcoholic improved, mortality index	22.31%	F_1 42.40% to F_4 17.14%	
11. Altered sex-ratios	109.60	♀ ancestors 86.50	—
12. Av. birth wt of litter	197.12	170.00	—
13. Av. individual birth wt.	77.16	70.35	—
14. Av. wt. 1 month old	228.64	213.94	—
15. Av. wt. 3 months old	425.11	404.13	—

The alcoholics have a higher proportion of their total mortality occurring very early, so that there is a great elimination of weak embryos and fetuses; this lowers their later or postnatal mortality to about the normal record.   In this case we have an elimination or selection of individuals or zygotes rather than a germinal selection.   The total mortality record for the experimented group is far higher than for the control and a greater percentage of abnormal young are produced.   The percentage of abnormalities is lower than in our former records, as is also the total mortality rate.   The improved mortality rate is partly due to better methods of breeding and caring for the animals.   Yet the mortality record of the alcoholic group is very high, and when corrected for the normal rate on the basis of the size litters concerned it becomes 189 against the control as 100.   Among the

normal animals of the same general stock as the alcoholics, not one grossly deformed individual has been born in over 400 cases, and, as stated above, this is a remarkable record which argues strongly for the perfection of the stock. In considering the defective young, one must also keep in mind the fact that these are not worse, but, on the contrary, are better organized than individuals which die during early stages of development.

At three months old, as No. 8 in the table indicates, fewer alcoholic than control animals were larger than usual or over size, though some were, while the next line shows that more alcoholic animals were small or under size, weighing less than 300 grams.

The later generations of the alcoholic stock are improved by the continued elimination of weak and defective individuals which die or are unable to breed, and also by the introduction of more and more normal germ plasm from generation to generation until a mortality rate of 42.4 per cent for the F_1 generation becomes only 17.14 per cent for the F_4 generation. This is a clear demonstration of the alcohol effect and may also serve to show the action of increased germ dosage. The earlier generations being nearer the directly treated animals receive higher doses than do the later generations where in most cases the dose has been considerably diluted by a mixture of normal germ plasm.

The sex-ratio in the alcoholic group seem to have been modified in ways which we have attempted to explain.

The average weight of the alcoholic litter is less than the normal and the average individual birth weight of an alcoholic specimen is also less than for the normal. The average weight of the alcoholic individuals at one month old is below the normal and the average weight at the age of three months, when guinea-pigs are about mature, is still below the weight of the control animals.

Therefore, in the fourteen measured points considered, the offspring of the alcoholic series are below the normal control in thirteen cases and apparently equal to the control in only one.

The qualities are largely the same as those we have considered

in former papers though analyzed in further detail.   They are also very similar to those recorded by Pearl ('17) in his table 14. From a physiological standpoint it seems to us that these qualities are all closely associated and finally come down to the three related qualities: ability to develop normally, grow rapidly, and live to maturity.   An animal possessing such qualities is usually termed a vigorous individual.   At present it can only be stated that these properties are due to the vigor of the germ cells from which the individual arose.   The qualities discussed might all involve a limited range of physiological factors so far as present knowledge permits a separation of such factors and they only show on the part of the alcoholics a reduced capacity of development and growth.   The same underlying cause may actually account for the abnormal sex-ratios, as has been pointed out in an earlier section.

Leaving the environment out of account, the normal development, growth and length of life of a zygote varies with the perfection or vigor of the germ cells from which it originated. An experimental treatment may act upon the germ cells of an animal so as to modify them in some general way which lowers their ability to react normally in combination with germ cells from another individual.   Thus zygotes are produced which tend to develop abnormally, grow slowly, or die during early stages of their existence, depending upon the degree of modification the treated germ cells have suffered.   We are fully embarrassed by the unsatisfactory nature of such statements, but have been unable to gather scientific facts that would permit any more definite estimate of the situation.

All of our experiments on the modification of the germ cells have given results which express themselves in some such general fashion.   Yet the germ plasm has been definitely modified and the subnormal condition is transmitted through a number of generations beyond the animals directly treated.   This result is original on the complex material used, and is of primary importance, although it may be disappointing in that it has not shown a modification in the mode of behavior of some particular character known for its Mendelian inheritance.

The experimental modification of the inheritance of definite characters by a treatment of the germ cells is a future possibility. It must be recognized, however, that one is able to produce grotesque monsters by a treatment of eggs or spermatozoa, and yet all of the known characters which Mendelize in such an individual may be expressed in a perfectly normal fashion. This may be due to the fact that comparatively few such characters are known. Aside from the future definite modifications of inheritance, it would seem from the present study that the 'general qualities,' for lack of a more suitable term, of an organism may be affected, on account of an experimental modification of the germ plasm from which it arose. The modification may have taken place several ancestral generations ago. This is really the inheritance of pathological conditions which were induced upon and transmitted by the ancestral germ plasm. Such a type of inheritance is no doubt important in its relation to the normal processes of development and inheritance.

15. GENERAL CONSIDERATIONS

A discussion of the literature bearing on the influence of various chemical substances on the egg and spermatozoön has been given in former papers of this series, particularly Stockard ('12 and '13). In all cases only the effects of the treatments on the zygotes immediately resulting from the modified spermatozoa or eggs have been studied. There has been no experimental investigation of later generations arising from the affected specimens. And indeed, in almost all cases the developing individuals were lost during early embryonic stages as in the X-ray experiments of Bardeen and the radium studied of Oskar Hertwig which are the most satisfactory investigations on the direct injury of the sperm. These experiments really supplied no available material for an investigation of the inheritance or transmission of the induced defective conditions.

Since the beginning of the present experiments other studies have been recorded which bear more directly on the results considered in the foregoing pages. Of particular interest in connection with our supposed differential effects of the alcohol treatment on

the behavior of the X and Y groups of spermatozoa is the ingenious double-mating experiment of Cole and Davis (14) with rabbits.   They found that when two male rabbits were mated with a single female, superfetation occurred in most cases, so that part of the resulting litter of young were sired by one male and part by the other.   The males differed in their fertilizing abilities, so that one more often sired the majority of young of a given litter, and in the total number of competition matings he sired the greater number of young.   This male with the fertilizing advantage was then treated for a month or more with the fumes of alcohol by the inhalation method.   As a result of this treatment his spermatozoa became affected in such a way that mated in competition with the same male he normally had beaten he now failed to sire any young.   Yet when mated singly or alone with a female he still possessed the power to beget offspring.   This is a striking illustration of the debilitating effect of a short alcohol treatment on the physiological behavior of these spermatozoa, thus lowering their fertilizing ability below that of other spermatozoa which were formerly less potent than they.

When it is seen how definitely and readily alcohol treatments affect the behavior of the spermatozoa, we are led to speculate as to whether the treatment might not affect the X and Y groups of sperm differently, and thus be partially responsible for a distortion of the sex-ratios, should such occur.   This responsibility may be due in the first place to a lowered fertilizing power on the part of one group of spermatozoa, thus giving rise to fewer individuals of one sex than of the other.   Or, in the second place, even though both groups of spermatozoa should be equally capable of fertilizing the eggs, one group might be more affected as to its ability to produce viable zygotes in combination with normal ova, and thus an early differential sex mortality would occur causing a modification of the proportion of one sex to the other among the young born.   We have elaborated somewhat on these possibilities in the section devoted to the sex-ratios of the alcoholic guinea-pigs.

Cole and Davis originally devised their experiment as a cru-

cial control for the influence of alcohol treatment on the male germ cells. In mating two males to a single female any defective condition that might arise among the offspring from one of the males, as compared with those from the other, could not be attributed to differences in developmental environment or in the qualities of the ova, as might possibly be the case where different females are used.

Cole and Bachhuber ('14) have employed the same method in a study of the effects of lead on the germ cells of the male rabbit and fowl. Their conclusion in regard to the rabbit is "that the offspring produced by male rabbits which have been poisoned by the ingestion of lead acetate into the alimentary tract have a lower vitality and are distinctly smaller in average size than normal offspring of unpoisoned males." This conclusion is in exact accord with the conditions shown by our F_1 generation of guinea-pigs sired by alcoholized fathers. Cole and Bachhuber have not reported on the transmission of the effects to later generations.

Their results with fowls "are interpreted as indicating that in fowls also poisoning of the male parent with lead results in offspring of a distinctly lower average vitality." This again accords with the results on the offspring when male guinea-pigs are treated with alcohol.

A later more extensive report concerning the influence of lead as a substance producing blastophthoric effects is given by Weller ('15). This investigator has treated both male and female guinea-pigs with commercial white lead. The lead is administered by mouth in gelatin capsules, the same method as was employed by Cole and Bachhuber ('14). The effects from the lead poisoning on the guinea-pigs are very similar to those obtained by treating the rabbits and fowls. Weller has been careful not to overdose the animals and his precautions would make it seem probable that any effect from the treatment which might be shown by the offspring was actually due to the lead poisoning and not to impaired nutrition or other indirect causes.

His conclusions are based on a total of ninety-three matings yielding 170 offspring. There were thirty-two control matings

which produced only fifty-eight offspring.   Whether or not every mating gave offspring is not definitely stated, but if so the average-size litter was unusually small, being only 1.81.   This would indicate either a stock of very low productivity or a high proportion of absorbed embryos and partial abortions, as a final result of which the litters would be small.   In the foregoing tables where the numbers of matings and young are very much greater, not one group shows so small an average litter.   From the thirty-four matings of lead-poisoned males with normal females, sixty-five offspring resulted, an average litter of 1.91, and from twenty-seven matings of normal males with lead females forty-seven young were born, an average litter of only 1.74.

The fact that among the few individual litters recorded there were three cases of litters of four, and five cases of litters of three, makes it seem as though there may have been a high proportion of mating failures, giving rise to the small average litters obtained when the total number of young is divided by the total number of matings.   The distribution and cause of these mating failures, as is pointed out in the text above, may be of considerable importance.

Weller has analyzed his results in some detail.   He takes into account the influence of litter size on the birth weight and gives several individual mating records which illustrate the effects of a treated sire on the birth weight of the young from a normal dam.

Weller has also taken into account the relationship between lead dosage and birth weight of the offspring without finding very consistent correlations.   The relationship between germ dosage and the condition of the offspring in our records may be calculated for every individual born in the alcohol experiments, yet the result is uninstructive so far as at present studied. There are a great number of confusing factors involved in this seemingly simple proposition.

Weller's final conclusions from the study of lead poisoning closely accord with our previous statements regarding the influence of alcohol on the same animals.   He finds that chronic lead poisoning in guinea-pigs produces a definite blastophthoric effect.

This can best be demonstrated upon the male germ plasm, in which case the blastophthoria manifests itself in some instances by sterility without loss of sexual activity, by a reduction of approximately 20 per cent in the average birth weight, by an increased number of deaths in the first week of life, and by a general retardation in development such that the offspring of a lead-poisoned male remains permanently under weight.

These experiments with alcohol and lead on rabbits, fowls and guinea-pigs seem to their authors to modify the male germ cells in a definite manner. The offspring sired by treated fathers are inferior to those from control males. The transmission of the defects to subsequent generations has not been reported.

In addition to the experiments on the direct treatment of the spermatozoa of lower forms, a few attempts have been made to treat the spermatozoa of higher animals directly with certain chemicals. Ivanov ('13) has given a short note on the effects of immersing the spermatozoa of several mammals in solutions of alcohol. He finds that when fertilization is obtained after such treatments a normal development follows and normal offspring are produced. To anyone who has studied the action of alcohol on the free swimming spermatozoa of lower vertebrates such results are not surprising. The most probable explanation is that the spermatozoön has been entirely protected from the action of the alcohol of the strengths used. When any action is obtained the usual effect on the spermatozoön is to render it immobile. To obtain a fertilization the motionless sperm must be activated by the use of some alkaline substance, such as NaOH. Following this activation the spermatozoa may often give normal offspring after union with normal ova, thus indicating that their chemical nature has not been disturbed. It is most difficult to treat the spermatozoön even of the very hardy fish, Fundulus heteroclitus, in such a manner as to injure it and afterwards obtain a fertilization. Dr. Wilson Gee ('16) experimented on the spermatozoa of fishes at Woods Hole for two seasons and found that the difference between an effective alcohol dose and a fatal dose was so slight that it required the most delicate adjustment of solutions in order to injure the spermatozoa to such a degree that the development of eggs subsequently

fertilized was rendered abnormal. Ivanov's report is certainly not sufficiently detailed to satisfy one that his results have any bearing on the problem of the modification of the germ cells by chemical treatment.

There can be no doubt that if a spermatozoön is actually affected by a direct chemical treatment, the egg which it fertilizes will develop more or less abnormally. The radium and X-ray experiments of Bardeen and Hertwig, as well as fertilization by foreign spermatozoa give conclusive evidence on this point.

The statistical research by Elderton and Pearson ('10) has frequently been quoted as if it shows that parental alcoholism was really to some degree beneficial to the human offspring. Their mathematical calculations were based on two series of statistics, the "Edinburgh Charity Organization Society Report and a manuscript account of the children in the special schools of Manchester provided us by Miss Mary Dendy." "Suspected drinkers were included with drinkers," "the parents could be divided into two classes only, those who are temperate and those who are intemperate," and many other such statements make this biological data somewhat unsatisfactory to those interested in an experimental modification of the germ plasm. These authors, however, do not claim to find any effect, either good or bad, of alcoholism on the offspring, and finally state that

On the whole the balance turns as often in favor of the alcoholic as of the non-alcoholic parentage. *It is needless to say that we do not attribute this to the alcohol, but to certain physical and possibly mental characters which appear to be associated with the tendency to alcohol.*[1]

Such a conclusion on the part of the authors themselves would scarcely warrant anyone else in claiming that an effect of alcoholism on the parent had given evidence of its existence in the quality of the children produced. A number of English physicians interested in alcoholism largely from a social and sentimental standpoint opened a bitter attack on the memoir by Elderton and Pearson, not because it claimed a beneficial effect,

[1] Italics are ours.

but merely because no harmful effect was shown. Such criticism is of little interest, yet one very serious point was cited against the data on which this study was based, and Pearson and Elderton ('10) in their reply failed to satisfy the objection. The children considered were in the neighborhood of nine years old at the time the statistics were collected and the fact that some parents were drinking at this time might not necessarily prove that they were drinking nine or ten years ago when the children were conceived. It is very evident that from our standpoint accurate data relating to this particular fact is most essential.

This study really has no bearing in the literature on the chemical modification of germ cells or the developing embryo, as Elderton and Pearson themselves state in the italicized portion of the quotation cited above. No one can confidently affirm that in their data alcoholics are being compared with normals or really whether any alcoholics or normals as such are actually being considered beyond the chance probability that some individuals of both classes creep into the statistics to be included in the two groups arranged.

Very recently Pearl ('17) has published a most thorough analysis of the influences of parental alcoholism on the progeny of the domestic fowl. He states (p. 285):

that a careful study of the present results makes it impossible to assert that the treatment of the parents has had no effect upon the progeny. . . . . The offspring of the alcoholists, as a class, are indubitably differentiated from the offspring of the non-alcoholists.

Such a statement agrees entirely with our results from the alcoholic guinea-pigs. In detail, however, Pearl finds that after treating fowls with alcohol the progeny produced are in some respects superior to 'the control. This, he believes, is brought about by an elimination of all weaker germ cells through the action of alcohol which thus serves as a selective agent to improve the race. At first sight this would seem to be entirely contradictory to our results, since the guinea-pig progeny is decidedly the worse for the experimental treatment. Yet the treatment in both cases has affected the progeny through its

action on the germ cells.   This is the point of actual importance and the one of chief interest from the standpoint of these experiments.   We are not here studying the alcohol problem from a social standpoint and it is immaterial whether the progeny be benefited or injured by the treatment of parental generations. Our interest lies in whether or not the germ cells are modified by the chemical treatment and whether the modification is of such a nature as to alter the qualities of the individuals which may compose the subsequent generations.

Pearl, of course, fully agrees with such a position, and states ('16 a, p. 258):

Our results seem to me to be supplementary to those of Stockard, and to throw an interesting light on the need for caution in reac ing a correct interpretation of all experiments in which a mildly deleterious agent acts upon the organism.

He also believes that his results are in no way contradictory to ours, but recognizes the fact that, although the same chemical substance may act upon the germ plasm of two different classes of animals, the visible response on the part of the animals need not necessarily be the same.   In other words, one is not always within the realm of legitimate scientific speculation who assumes that since a given substance acts to induce a certain response on the part of one animal species that the same substance will call forth a like response on every other species.   "What is one man's food is another man's poison."   With this we fully agree; it is dangerous to draw universal deductions from experiments on any one or two classes of animals.

Another possibility also recognized by Pearl presents itself in considering the opposite effects of the alcohol treatment on the progeny of guinea-pigs and fowls.   Small doses of many substances, one of which is alcohol, may form a physiological standpoint produce a stimulating effect, while larger doses produce decided depression.   There is a possibility that the same may be true of the action of such substances on the germ cells.   Pearl has discarded such an explanation after very fair consideration, and is possibly right in so doing.   The experiences, however, with the guinea-pigs makes our opinion decidedly prejudiced in

favor of the possibility, that although a sufficiently large dose may have been used, yet it did not act solely to eliminate germ cells as such, but also caused the production of many zygotes which died during early developmental stages.

The amount of dosage is very important. Treating female guinea-pigs with considerable doses of alcohol fumes only shortly before and during their pregnancies certainly does not injure the offspring to any noticeable degree. While the same dose of treatment, if administered for a number of months or years, will render these mothers almost incapable of producing vigorous young, even when mated with normal males.

Pearl ('17, p. 281) finds regarding his 1915 results which were obtained after the treatments had been running for only a few months that considering the number of animals in the experimental series the individual differences are not in every case sufficiently large to be significant in comparison with their probable errors. The control in this case was also not what Pearl had wished. He had originally chosen a carefully pedigreed control, taking as the one control male a half-brother of the three experimental males and using control females that were sisters of the treated hens as recorded in table 5, p. 158 ('17). The only control male, No. 666, proved to be practically sterile and useless. This necessitated the use in paper No. III of an ordinary random sample control instead of the refined control originally planned in Part I of the series of papers, and nullified the statement in the summary of Part I, p. 162, that "Full brothers and sisters of treated are used as control."

For certain qualities, such as the fertility and hatching records of the eggs, the control was not in all cases the same cross as the experiment, which was invariably between Barred Plymouth Rock hens and Black Hamburg cocks. The hatching weight and rate of growth of the experimental chicks on account of want of control data from the 1915 season were compared with chicks from a similar cross hatched and reared in 1913. Different keepers were in charge of rearing the chicks during the two different seasons. These unfortunate conditions, all of which are pointed out with conscientious fullness by Pearl, make it rather difficult

to fully estimate the actual significance of the differences between the experimental offspring and the control groups used.

Fortunately, however, the data from the 1916 season is available (Pearl, '16 b) for comparison with the 1915 results. The alcohol treatments were continued throughout the time so that the 1916 chicks are derived from more highly alcoholized parents. Should the alcohol continue to improve the race by "completely putting out of commission all of the weaker germ cells," the 1916 results should in all respects show a further improvement in the qualities that had been previously benefited.

The percentage of infertile eggs given in the 1915 table may be reversed to per cent of zygotes formed and compared with this column in the 1916 table. The percentage of zygotes formed in the several combinations of alcoholic mating should be less than in 1915, and they are. When both parents were alcoholic in 1915, 40.8 per cent of the eggs formed zygotes, while in 1916 only 21.95 per cent produced zygotes; sire only alcoholic, 74.8 per cent zygotes in 1915 and only 53.52 per cent in 1916. This is in line with the lowered fertility and increased number of mating failures from the alcoholic guinea-pig records. The more decidedly alcoholic the guinea-pigs become, the smaller the litter size from double alcoholic and sire only alcoholic matings, and the greater the number of failures to conceive.

With the guinea-pigs, however, this is not alone due to a destruction of weak germ cells by the treatment, but is certainly in part due to an increased very early prenatal mortality for which much evidence is given in the body of the present paper. The smaller number of zygotes formed by the treated fowls is probably also due in some cases to death in very early stages, as blastulae or gastrulae, before the egg is laid; or in the hen's eggs these weakened zygotes may not be able to withstand the developmental interruption following the laying of the egg. Embryos dying during such stages could not be identified except by a most minute study.

It seems to us in keeping with what is known of biological reactions in general and the guinea-pig histories in particular to take the following position. The alcohol treatment acts on the

germ cell populations of both fowls and guinea-pigs in such a manner that the weakest or least resistant ova and spermatozoa die from the effects of the treatment as germ cells without taking part in zygote formation. The somewhat more resistant ova and spermatozoa are greatly injured though still capable of forming zygotes. The zygotes, however, are so defective as to be capable of only a short period of development and die during stages too early to be definitely detected by gross examinations of either the fowl's egg or the mammalian mother. Still other embryos are capable of development to later stages and are actually found dead, not only as the youngest embryos to be identified, but from these early stages there occurs a continuous series of prenatal deaths up to the full-term still-births. Immediately after birth the postnatal mortality is greatest and gradually decreases until those specimens capable of reaching maturity may often enjoy a comparatively long life.

At the present stage of the two experiments it would seem as though this elimination of defective germ cells and very early embryos was much more intense in the fowls than in the guinea-pigs as a group; so that the late prenatal and postnatal mortality among the fowl progeny was low and those specimens that hatched were the hardy survivors from this early vigorous process of germ cell and individual selection. The records from the double alcoholic and male treated lines among the guinea-pigs forms a second step. The size of the litters and failures to conceive in these lines indicates a rather high degree of infertility or germ cell debility as well as early prenatal deaths, though this is not so extreme as among the fowls, and the late prenatal and postnatal mortality is higher.

Finally the female treated guinea-pig lines produce large litters and have few infertile matings, indicating a low germ cell and early prenatal mortality, and here the late prenatal and postnatal mortality is highest, not entirely on account of the action of the treatment on the developing individual in utero, since the same condition is found among other female generations than the one directly treated.

This presentation of the situation is somewhat similar to that

which Pearl ('17) has illustrated in his diagrams, figures 5 to 7, pages 290 and 291. The chief difference being that we would decrease the proportion of eliminated germ cells and increase the proportion of defective and non-viable zygotes, and thus emphasize the selection of individuals rather than of germ cells.

A further consideration of Pearl's 1916 results as shown in table 1, p. 676 ('16 b), may be used to argue in favor of our position. The 'prenatal mortality' column of this table when compared with the 'dried in shell' column from 1915 records (table 1, p. 244, '17) should show lower percentages according to our interpretation of Pearl's expectation for an improved stock from the alcoholic lines. Instead of this, in only one combination is the prenatal mortality lower. In both parents alcoholic it has been lowered from 26.9 per cent to 11.11 per cent, and here the postnatal mortality as we would expect is increased. In the other cases dam only alcoholic, none of which were reported for 1915 on account of the useless control male, gives 80 per cent prenatal mortality sire only alcoholic increased to 47.08 per cent from 36.6 per cent; sire and one grandparent, 46.84 per cent; one or more grandparents, 46.02 per cent; all alcoholic ancestry, 45.95 per cent, which is a considerable increase over the 1915 records. The control of 1916 also shows a higher prenatal mortality than that of 1915, though it is not stated whether the same breed crosses are used in the two controls.

The postnatal mortality of the 1916 control is, on the contrary, lower than the postnatal mortality of the twenty-two 'random sample matings' of 1915.

While the total mortality for all the alcoholic groups is about the same, 17.6 and 16.5 per cent, for the two seasons, the individual combinations show wide variations. From both parents alcoholic the 1915 postnatal mortality was 10.6 per cent, while for 1916 it rose to 25 per cent, sire only alcoholic fell from 21.1 per cent, 1915 record, to 13.79 per cent, 1916 record. Sire and one grandparent alcoholic gave a postnatal mortality of 28.38 per cent, while the non-alcoholic postnatal mortality was 21.2 per cent.

Considering the numbers involved, the records from the prog-

eny of the 1916 matings after longer alcohol treatment do not seem altogether improved as compared with the 1915 records. A comparison of individual lines in the tables frequently show disadvantages for the 1916 matings. This would seem as though some injured zygotes were present and all of the affected germ cells had not been completely eliminated by the treatment. The percentage of abnormal specimens among the 1916 alcoholics is about the same or slightly more than among the control, while Pearl had counted this point in favor of the alcoholics from his 1915 records.

It would thus seem, as Pearl ('17, 292) himself suggests, that "it might be supposed that with larger administration to the fowls (higher germ dosage) or more years of drinking behind them in the case of Elderton and Pearson's workingmen, the conditions shown in figure 7 would gradually pass over into those shown in figure 5." That is, that not only weak germ cells would be eliminated by the treatment, but that also a considerable proportion of defective individuals would arise to be eliminated during various developmental stages or persist as degenerate specimens. From these conditions we believe that there is a really close agreement between the results on the fowls and the guinea-pigs.

These suggestions are advanced only in a spirit of the most friendly criticism. We have worked long enough in accumulating and considering evidence bearing on the various phases involved in this problem to highly appreciate the masterly manner in which Pearl has considered and analyzed his data; and we are thankful for many suggestions that have come to us through the contribution on parental alcoholism in the fowls. In the end our aims and objects are the same, to affect the germ plasm in so definite a manner as to be able to predict the quality and degree of the modifications subsequently expressed in the generations to follow.

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A DEMONSTRATION OF THE ORIGIN OF TWO PAIRS OF FEMALE IDENTICAL TWINS FROM TWO OVA OF HIGH STORAGE[1] METABOLISM

OSCAR RIDDLE

From the Station for Experimental Evolution, Cold Spring Harbor, L. I., N. Y.

THIRTEEN TABLES

Much has been written on identical twins; but, assuming that it is a fact that such twins arise from the first two blastomeres of a single ovum, not a single fact seems to be known concerning either of the first two questions which one is tempted to ask concerning the germs from which such twins arise. Probably the first of these questions is, If identical twins arise from two separated blastomeres, why do the blastomeres separate in these special and particular cases? Second, What functional differences characterize two such ova that produce male twins in one instance and female twins in the other? It is possible that the data presented here do not supply us with a fact concerning the reason for the separation of the two blastomeres in these occasional instances. A suggestion on this point is offered. But the present data do, beyond question, give us a fact concerning the functional status of two particular germs which produced two pairs of female identical twin ring-doves.

In my earlier studies[2] on the eggs (yolks) of doves and pigeons it was learned that males arise from eggs (yolks) of lesser storage metabolism (small size, and higher metabolism), and females from eggs (yolks) of greater storage metabolism (large size, and

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[1] High storage metabolism is to be interpreted as low (oxidizing) metabolism.

[2] See, a) Science, N. S., vol. 35, pp. 462–463, March 22, 1912; b) Carnegie Year Book, no. 12, p. 322, 1913; c) Bulletin of the American Academy of Medicine, vol. 15, no. 5, pp. 265–285, October, 1914; d) American Naturalist, vol. 50, pp. 385–410, July, 1916; e) Journal of the Washington Academy of Sciences, vol. 7, no. 11, June 4, 1917; f) Science, N. S., vol. 46, pp. 19–24, July 6, 1917.

lower metabolism). I have now obtained two cases of female identical twins, and am able to know that the ova (yolks) which produced both of them were extraordinarily and abnormally large.

We may first note that these two instances supply a strong confirmation of my earlier conclusion concerning the correlation of high-storage yolk values and femaleness. Next we may examine the data which demonstrate that the twin-producing yolks were of exceptionally large size.

SIZE OF EGGS AND YOLKS IN RELATION TO THE TWO CASES OF TWINS

The yolk of a dove's egg cannot, of course, be directly weighed on a balance and then be incubated with the hope of producing young.   But, as we shall see, the two procedures on the same egg are unnecessary to the demonstraiton that the twin-producing yolks were of extraordinary size.   Again, the weights of two entire eggs (yolk + albumin + shell) from the same bird, and the same clutch, may be different and yet this difference not indicate which egg contains the larger yolk (ovum).   But the amount which two such eggs may thus differ, without showing the direction of difference in the yolks, is limited.   And, for the species under consideration, as well as for several others, the limits of such difference are now approximately known.   Accurate direct weighings of nearly 15,000 yolks of various pigeon species and hybrids have been made.   Probably nearly 4,000 of these are eggs of the species which produced these two instances of twins. Certain aspects of these data will be utilized here for the purpose just indicated.

One of the twin-producing eggs was the seventy-sixth egg laid by that particular female.   The other twin arose from the fiftieth egg of another female.   In our main study the complete egg-laying history is kept of some hundreds of doves and pigeons. We are here able, therefore, to give the exact weight of every egg produced by each of these two twin-producing females prior to, as well as after, the appearance of their twin-producing eggs. For the blond ring female (No. A248) this record is given com-

plete in table 1. The similar record of the hybrid[3] female (No. 60) is given in table 2. A glance at those two tables will show how conspicuously larger is each of the twin-producing eggs than is any other egg of the series to which it belongs; the tables give the necessary details for one hundred[4] eggs of one series and ninety-seven in the other.

The extraordinarily large size of the eggs which produced the twins, in comparison with all of the other eggs produced by these particular parents (totals of 116 and 134 eggs), is itself sufficient to make it extremely probable that the yolks contained within them were of very large size. The certainty of their large yolk size becomes apparent in the light of the results of our accurate measurements on many thousands of eggs. It is, of course, impossible to give all of these latter measurements here; this is also unnecessary since these will appear in connection with the complete account of our studies on sex in pigeons. It does seem necessary, however, to give here the particular segment of this evidence which is contained in the several following tables.

Reference to tables 5 and 6 will show that the eggs which produced the twins were, in one case, 24.9 per cent, and in the other, 43.1 per cent larger than the associated egg (of the same clutch). Such amounts of difference between the two eggs of the clutch are highly abnormal; except in cases of evident dwarfing of the smaller egg of the pair they practically do not exist. In the two pairs of twin-producing eggs it will be observed that the smaller eggs of the pairs are not dwarfed. A number of cases approximating to these extreme differences have, however, appeared in our records. An assistant has gone through the entire body of our breeding records and listed all pairs of eggs in which the members of the pair differ by 20 per cent or more (tables 7 to 12). In connection with these summaries it was thought advantageous

[3] This female is hybrid between two very closely related ring-doves—Streptopelia alba and St. risoria ($\frac{7}{8}$ alba, $\frac{1}{8}$ risoria).

[4] Sixteen additional eggs (to December 1) have since been laid by female A248; the largest egg weight among these is 9.40 grams. Thirty-seven eggs have since been added to the series containing ninety-seven eggs, and the largest egg weight among these is 9.11 grams. Most of the tables of this paper are summaries of data collected to April, 1917.

to list all pairs of eggs (of whatever per cent of difference in weight) in which the yolks weights had been obtained and were found to differ by as much as 35 per cent. This latter amount is likewise abnormal for yolk weight differences; usually, this difference (in pure species) is only about 9 to 15 per cent. Since the egg weights of incubated eggs are thus included, the lists as given contain all of the strikingly abnormal or ill-matched pairs of eggs that have been encountered among the nearly 20,000 doves' eggs that we have studied. These selected data are partially classified in the tables (7 to 12) according to the kind of female which produced the eggs.

Several of these pairs of disproportionately sized eggs have been incubated and the sex of the resulting offspring learned; these data are also fully given in the tables. Sex is certainly correlated with the size, or storage metabolism, of the ova (yolks); and, in pure species, both of these are certainly correlated with the order of the egg in the clutch, as has also been pointed out in earlier publications already cited. In hybrids, however, and most markedly in the hybrids from the wider (generic) crosses, any regularity of the presence of smaller yolks in the first eggs of the clutch is lost. At the same time a high predominance of males from the first and of females from the second eggs of the clutch is also lost. Some instances of these conditions will be observed in the tables. But, as we have previously pointed out, there are conditions other than yolk size which also influence the sex that is to proceed from a particular yolk. A sperm from a different genus, or subfamily, may cause a male to arise from an ovum which, if fertilized by its own species, would have produced a female.

Further, there is at hand considerable evidence in favor of the following interpretation: The sperms formed by hybrids, particularly by generic, subfamily, and family hybrids, are of the most varying degrees of fertilizing power. That is to say, the sperms produced by a particular male vary thus, and these sperm differences are probably not devoid of power to influence both the degree of development and the sex of the offspring from the ovum with which the sperm unites. In still other words, differ-

ent sperm from the same hybrid male may exercise opposite tendencies for the production of sex.[5]   A probable instance (Q of 265) of this is partially described in the footnote to table 8.

The females which produced the twins, and the eggs listed in tables 1 and 2, were mated to blond ring-dove (Streptopelia risoria) males.   Indeed, these males are sire and son; and, further in one of the series the sire is mated to his daughter (A248). The hybrid female (alba-risoria No. 60) is thus mated with one of her parent species, and the two species which enter into her composition are closely related ones.   In consequence both of these female tend—aside from special modifying conditions— to throw higher proportions of males from first eggs of the clutch and of females from the second eggs of the clutch (tables 3 and 4). The bisexual clutches (those producing the two sexes) are considered in tables 5 and 6.   Among these latter this proportion is 5 : 1, or 5 : 3 (?) for female A248,[6] and 12 : 7 for female 60.

Reference to table 7 will show that all pairs of eggs obtained from pure blond rings which differed by as much as 20 per cent in weight had yolk weights which differed in the same sense as the egg weights; i.e., in this pure species, the yolk of the second egg was invariably (seven cases) larger than the yolk of its clutch mate, when the total weight of the second egg was 20 or more per cent larger than the egg weight of the clutch mate.   In the case of the twin-producing egg belonging to this series the egg-weight difference was 43.1 per cent; this very wide difference fully guarantees the larger size of the yolk which it contained and which gave rise to the twins.

The difference (43.1 per cent) is also far greater than that of any of the thirty-five pairs of yolks weight of alba-risoria hybrids given in table 8.   That table shows that every egg-weight difference of more than 14 per cent correctly indicated the direction of the difference between the pairs of yolks produced by these

[5] The factual support of this unorthodoxy must, after consulting the papers cited under note 2, in part await the publication of, a) C. O. Whitman, Posthumous Works, vol. II (The Carnegie Institution, in press) and, b) our own forthcoming work.

[6] Some evidence from inbred relatives of this bird possibly indicate a slight contamination of *St. alba* in this female.

hybrids. In only one case of the thirty-six clutches listed—
one in which the difference in egg weight was only 13.9 per cent—
did the difference in egg weight fail to show which egg contained
the larger yolk. This failure, moreover, concerns the first pair
of eggs laid during the life of the bird, and this has long since
been observed to be a clutch in which the usual order, both of
sex delivery and of yolk size, is more often disturbed or reversed.
It may be incidentally noted that the first clutch produced after
prolonged reproductive rest is similarly disposed—even in pure
species—to supply a large yolk to the first egg and a small one to
the second egg, and to reverse the normal order of the resulting
sexes in the same sense.

Table 8 will show that the other twin-producing egg, that of
hybrid female No. 60, was an extraordinarily large egg when
compared with the extraordinarily large eggs produced by any
and all of our hybrids of similar or related kinds. Only three
eggs of the fifty-two pairs from all similar sources equaled it
in size. The data of the table leave no doubt that this twin-
producing egg contained a yolk of unusually large size.

The effects of hybridity in the female on the regularity of
delivery of larger eggs and yolks in second eggs of the clutch (as
indicated by the segment of data contained in the several tables)
may be summarized as follows:

Pure St. risoria.................. {Eggs   2nd larger, 20; smaller, 0} (table 7)
                                   {Yolks, 2nd larger, 7; smaller, 0}

Pure St. alba and T. orientalis.. {Eggs, 2nd larger, 6; smaller, 0} (table 9)
                                   {Yolks, 2nd larger, 2; smaller, 0}

Risoria-alba hybrids............. {Eggs, 2nd larger, 48; smaller, 4} (table 8)
                                   {Yolks, 2nd larger, 48; smaller, 4}

Miscellaneous hybrids........... {Eggs, 2nd larger, 14; smaller, 4} (table 10)
                                   {Yolks, 2nd larger, 11; smaller, 5}

Common pigeons................ {Eggs, 2nd larger, 2; smaller, 2} (table 12)
                                   {Yolks, 2nd larger, 1; smaller, 3}

Alba-orientalis hybrids (generic {Eggs, 2nd larger, 27; smaller, 35} (table 11)
  hybrids)..................... {Yolks, 2nd larger, 26; smaller, 34}

It is clear, therefore, that these data justify the preceding statement on the effects of hybridity upon the normal size relations of the two yolks (ova) of the pigeon's clutch. They also emphasize the fact that the two twin-bearing eggs were from bird groups which assuredly throw a very high proportion of larger yolks in the second egg of the clutch. Both twin-producing yolks were, as already noted, the second of the clutch.

The two series of breeding records which supplied these two cases of twins (tables 1 and 2) afford an opportunity partially to illustrate still another condition which affects the sex production, and to a certain extent the storage capacities, of the ova of pigeons. In earlier papers we have referred to 'crowded reproduction' merely as an aspect of 'reproductive overwork.' We can here particularize to the extent indicated in tables 1 to 4.

The summaries at the bottom of tables 1 and 2, and the four divisions (columns 2 and 3) of tables 3 and 4, show that the sex ratio changes with the rate at which the eggs are produced. In the case of ♀ A248 (table 1) those clutches which were separated from the preceding clutch by an interval of eight days or more yielded 26 ♂ : 13 ♀ (sex unknown 9); those of seven-day intervals, 9 ♂ : 23 ♀ (unknown 8); and those of six-day intervals, 4 ♂ : 8 ♀ (unknown 0). For the hybrid ♀ 60 (table 2), the corresponding figures are: 26 ♂ : 27 ♀ (unknown 8); 5 ♂ : 11 ♀ (unknown 2); 2 ♂ : 9 ♀ (unknown 5). Under the most crowded reproduction (six and seven days) in these two series it is clear that there is an undoubted deficiency of males, even if all of the eggs of unknown sex value were classed as males. The fact that one pair of twins arose from a clutch with a six-day interval (table 1) and the other from a seven-day interval (table 2) is therefore significant for the purposes of the present paper. The actual time intervals involved predisposed, so to speak, these clutches to femininity, and both our published and unpublished data show conclusively that femininity is correlated with a high storage metabolism of the ova.

Tables 3 and 4 supply still a different method of analysis of the relation of 'crowded reproduction' to sex. In column 2 of those tables the whole period of egg laying (shown in tables 1 and 2)

is divided into its natural divisions; i.e., into the actual periods of
work and rest of the female parent.   It will be observed that both
females threw highest proportions of male offspring from the
longest clutch intervals and fewest from the shortest intervals.
In the eight instances there is not an exception.   Moreover,
when each of these natural periods is subdivided into a first and
last half and the clutch intervals and the sex ratios are calculated
anew (columns 2 and 4, tables 3 and 4), the same fact is again
demonstrated.   When the undivided natural periods are put
alongside the sex ratios, calculated as percentages, the figures
speak for themselves:

♀ A248	♀ 60
16.5 da. = 2♂ : 1♀ = 33.3% females	14.5 da. = 12♂ : 10♀ = 45.5% females
7.6 da. = 16♂ : 10♀ = 38.5% females	9.9 da. = 10♂ : 10♀ = 50.0% females
7.4 da. = 17♂ : 17♀ = 50.0% females	7.7 da. = 6♂ : 15♀ = 71.4% females
7.0 da. = 4♂ : 16♀ = 80.0% females	7.1 da. = 4♂ : 11♀ = 73.3% females
Twin from six-day interval	Twin from seven-day interval

In this connection we may consider the question whether the
parents continued to produce eggs immediately after the twin-
producing eggs were laid and what was demonstrated as to the
sex ratios in these eggs.   Reference to table 2 will show that the
hybrid female (60) laid only one other clutch of eggs before taking
a rest (forty-four days).   We are therefore unable to say what
the sex ratio from this female would have been had she continued
'crowded reproduction.'   The period of rest is, of itself, however,
an evidence of the weakness (following overwork) which we have
learned to associate with a high proportion of females.   But even
the next complete reproductive period following this period of
rest yielded eleven females to four males, with two additional
embryos too weak to hatch.   In the case of the other twin-
producing parent (♀ A248, table 1) it will be observed that egg
laying was continued at regular seven-day intervals for twelve
clutches after the twin-bearing clutch.   From these eggs four
males and sixteen females were produced, and four eggs were too
weak in developmental power to permit us to know their pros-
pective sex.   Here one series of eight consecutive eggs produced
females; another unbroken series of five eggs produced females.

Unquestionably, in the parents which supplied a test of the matter, the twin-bearing egg was immediately succeeded by the production of a high proportion of female-producing eggs.

The question of variations in the relation of yolk weight to egg weight under the conditions of reproductive overwork and of 'crowded reproduction' is not answered by the data of the tables given here and lies outside the scope of this paper. For the present purpose, and by way of summary, it may be observed that both cases of twin-producing eggs occurred, a) in reproductively overworked females; b) in periods of 'continuous activity;' c) in very short intervals—six and seven days—since the preceding clutch, and, finally, that such crowded reproduction tends to produce an excess of females.

DOUBLE-YOLKED EGGS IN DOVES AND PIGEONS

There remains for consideration the possibility of the origin of the two cases of twins from 'double-yolked' eggs. Two sets of facts show that this did not occur. We may first note the conclusive data obtained from the twins themselves, and from the eggs that produced them, and later produce the record of the few cases of double-yolked eggs that have appeared in our studies with doves and pigeons.

The pertinent facts concerning the first pair of twins (from ♀ 60) are as follows: This egg was laid on March 7, and failing to hatch on March 22, was opened for examination. Two nearly full-term dead embryos were found; both birds were plainly smaller than normal birds ready to hatch, but both seemed practically completely formed and ready to hatch. A very considerable amount of yolk, however, remained unabsorbed, and both umbilici were plainly united at a nearly common point on the single yolk-sac. Both young were plainly females; in one of the two there was a distinct right ovary as well as the usual left ovary, but I was unable to make sure that a similar right ovary was also present in the other.

The facts obtained on the second twin-bearing egg (from ♀ A248) were as follows: When this egg was candled (held toward the light, as is done on the second to the fifth day for all eggs

incubated) to test its fertility on the third day of incubation, two embryos were plainly seen. It was then noted that the two were close together; that in moving, turning, or shaking the egg, they invariably turned together, and that their position with reference to each other could not be altered. Clearly they were contained within the same ovum. It was thus known in advance that this was a twin-bearing single yolk. On the fourteenth day of incubation—just before the young were due to hatch—the egg was opened so as better to learn the conditions presented by the twins. Two young were found, one dead, the other alive. The dead young was practically a full-term embryo, perhaps slightly larger than the live one which seemed nearly ready to hatch. There remained here also a considerable amount of unabsorbed yolk; and, as in the previous case, the umbilici had a practically common point of union on the yolk-sac. Both birds were plainly females[7] and both birds possessed right ovaries which were one-half as large as the left ovaries.

Five 'doubled-yolked' eggs have appeared among the approximately 20,000 doves' eggs that have been examined. The size of these eggs compared with the other egg of the clutch, and with the size of the eggs of the immediately preceding and succeeding clutches, is given in table 13. Four of the five cases occurred among the eggs of hybrids. The one case of a female of pure species (Stigmatopelia senegalensis) was supplied by a female which otherwise showed the following reproductive abnormalities: Two clutches immediately preceding the double-yolked egg were clutches of one egg each; previous to these she had laid fifteen clutches, fourteen of which consisted of two eggs. The double-yolked egg was the last egg produced during the year (November 25), and the last in life for this bird, except that an egg was present in her oviduct when she died three and one-half months (March 5) later.

The second of the double-yolks was produced by a female hybrid (alba × risoria) from her third egg in life. The two yolks of this egg were of most strikingly abnormal size—both together being

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[7] The sex can usually be definitely learned in nine- to ten-day embryos of those species whose incubation period is from fourteen to fifteen days.

little more than one-half the size of one normal yolk for birds of this kind. The immediately preceding eggs and the succeeding one were similarly much undersized. And, further, this female produced eggs at an abnormally slow rate during the entire year.

The third double-yolked egg was produced by a generic hybrid (T. orientalis × St. alba), whose reproductive record seems to be fairly normal for her kind. She was mated to another female when the double-yolked egg was produced.

The fourth and fifth of these double-yolked eggs were also produced by generic hybrids. The fourth was from a Stig. senegalensis × St. alba hybrid. It was produced only five days after a preceding clutch—a thing of most unusual occurrence; in another case she produced two clutches, of one egg each, only four days apart.[8] The fifth egg was from a complex generic hybrid (of three species, orientalis, risoria, alba), which produced eggs during only one season, and only three clutches after the abnormal one containing the double-yolk.

From the above records it seems clear, therefore, that double-yolked eggs of doves are practically restricted in their production to hybrids from wider crosses or to birds showing striking reproductive abnormalities or to both of these. The history of the few cases of double-yolks that are known would indicate, then, that such eggs would not be expected to appear in the series in which the two cases of twins were found.

ON THE CAUSE OF THE FORMATION OF IDENTICAL TWINS

At the beginning of this paper it was stated that on the basis of the present data a suggestion could be offered as to the reason for the occasional separation of the blastomeres which leads to the production of identical twins. Possibly the data do not really provide such a suggestion; but, knowing that the two eggs that produced two pairs of identical twin ring-doves were from yolks

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[8] These eggs may of course be considered as of the same clutch; in this case the abnormality would consist in the time interval being a four-day period instead of the normal forty hours.

of most extraordinary size, the writer can not but wonder if there existed a causal nexus between the extraordinary size, on one hand, and the unusual separation of the blastomeres on the other.

Plainly the main question is, Why, or by what means, is 'independent' development instead of coördinated, mutual, integrated development initiated in the two blastomeres? One means already known for obtaining this 'independent development' is that of physical separation of the blastomeres. Surely, the exact placement and position and inclination of the early blastomeres (meroblastic eggs) are not wholly out of reference to the size and to the consequent polar configuration. And surely the type of cleavage, normal to normal blastula-formation, etc., is not out of reference to the normal size and shape of the ovum. A somewhat unusual disposition of the segmentation spheres at the animal pole—these being, at their outer borders, abnormally raised in extraordinarily large eggs and abnormally lowered in extraordinarily small ones—would thus seem to afford a possible clue to this relatively rare occurrence.

In holoblastic eggs the egg-size might still be the conditioning factor, as in the case just noted of meroblastic eggs; for, although the blastomeres there are always in apposition, the centers of metabolic activity (nuclei, centrosomes, etc.) in abnormally large eggs would be separated to a degree unusual to the species, and thus conceivably afford a basis for the 'independent action' of the first two segmentation spheres. Abnormally small ova, in division, would provide two cells with abnormally (for the species) large surface areas in proportion to their masses, and conceivably this may similarly result in the immediate assumption of 'independent development' in each blastomere.

According to the view just sketched, identical twins should arise from the extremely large and the extremely small eggs of a species. Presumably such would be produced in approximately equal numbers. According to the theory of sex hitherto developed by the writer, males should develop from the smallest and females from the largest eggs. Apparently, the same size relations should hold for the sexes in twin-producing eggs. The two cases of identical twins described in this paper are two instances in sup-

port of both of the above-mentioned views. A few cases of identical male twins from extremely small ova of the pigeon would completely establish the view as stated for twinning in pigeons. Our present data undoubtedly establish the relations of yolk size to sex and give much warrant for the prediction that if identical male twins ever arise from the ova of doves and pigeons they will arise from small ova.

In conclusion, it may be emphasized that the correctness or incorrectness of the tentative hypothesis concerning the causes of twin-formation, as stated in the immediately preceding paragraphs, is unimportant to the main purpose of the present paper. The available data concerning the germs which gave rise to two pairs of female twins demonstrate that each pair arose from a single ovum and that each pair arose from an ovum of high storage metabolism.

TABLES

TABLE 1

Record of ♀ A248—nearly or quite pure St. risoria

SEX	CLUTCH	DATE	WEIGHT	NUMBER OR REMARKS	SEX	CLUTCH	DATE	WEIGHT	NUMBER OR REMARKS	SEX	CLUTCH	DATE	WEIGHT	NUMBER OR REMARKS
?♀	A1	3/7	6.775	Hatch	♀	R1	8/11	6.75	E301	?♀	H1	3/13	7.81	K370
	A2	3/9	7.870	Embr. (2)¹	♀	R2	8/13	7.46	E372	♂	H2	3/15	9.17	K377
♂	B1	3/18	7.097	A580		S1	8/21	7.28	Hatch	♂	I1	3/22	7.93	K352
	B2	3/20	8.280	Infer.	♀	S2	8/23	8.10	E358	?♂	I2	3/24	8.69	K372
♂	C1	3/28	7.125	A662	♂	T1	9/1	7.48	Hatch	♀	J1	3/30	7.18	Hatch
♂	C2	3/30	8.402	A587	♂	T2	9/3	8.15	E485	?♂	J2	4/1	8.51	Hatch
?	D1	4/6	7.312	Hatch	♂	U1	9/11	7.35	E486	♂	K1	4/7	7.43	K421
♀	D2	4/8	8.495	Hatch	♂	U2	9/13	8.35	E498	♀♀	K2	4/9	10.63	Twins
♀	E1	4/16	7.490	A712		*	*	*	*						
	E2	4/18	8.323	Embr. (2)	♂	V1	9/21	6.95	Hatch	♂	L1	4/16	8.13	K463
♂	F1	4/26	7.30	A759	♂	V2	9/23	7.64	E464		L2	4/18	8.89	Embr. (6-7)
♂	F2	4/28	8.20	A718		*	*	*	*						
♂	G1	5/5	7.07	A766	♀	W1	10/17	7.22	Hatch	♂	M1	4/25	8.22	K429
♀	G2.	5/7	8.28	A737		W2	10/19	8.31	Infer.	♀	M2	4/27	8.70	Hatch
♀	H1	5/13	6.96	A831		*	*	*	*						
♀	H2	5/15	8.55	Killed (14)	♂	X1	10/28	7.57	E434		N1	5/4	7.90	Infer.
♀	I1	5/22	7.64	A887	♂	X2	10/30	7.56	Hatch	♀	N2	5/6	9.05	K632
♀	I2	5/24	8.50	A900		*	*	*	*						
					♀	Y1	12/1	7.56	E564	♀	O1	5/13	8.13	K592
					?♂	Y2	12/3	7.56	Hatch	♀	O2	5/15	8.96	K541
					♀	Z1	12/11	7.63	Hatch	♀	P1	5/22	8.19	K676
						Z2	12/13	8.80	Infer.	♀	P2	5/24	9.01	K681

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Sex	Tag	Date	No.	Egg	Sex	Tag	Date	No.	Fate	Sex	Tag	Date	No.	Egg
♂	J1	5/30	6.77	A852	?♂	AA1	12/21	7.24	Hatch	♂	Q1	5/31	8.05	K701
♀	J2	6/1	8.29	A874	♂	AA2	12/23	8.56	Hatch	♀	Q2	6/2	9.03	K750
							1917							
♀	K1	6/7	6.99	A851	♂	A1	1/1	7.28	E525	♀	R1	6/9	7.94	K730
♂	K2	6/9	8.36	A872	♀	A2	1/3	8.47	E679		R2	6/11	9.00	Embr. (10)
♀	L1	6/15	7.04	E8	♂	B1	1/11	7.75	E638	♀	S1	6/18	8.01	K795
♀	L2	6/17	8.44	E68		B2	1/13	9.08	Infer.	♂	S2	6/20	8.70	K724
	M1	6/24	7.04	Infer.	♂	C1	1/21	7.49	Killed (15)		T1	6/27	7.83	Infer.
♂	M2	6/26	7.74	E2	♂	C2	1/23	8.51	Killed (14)	♀	T2	6/29	8.86	K807
?♂	N1	7/3	7.29	E234	♀	D1	2/1	7.50	Hatch	♀	U1	7/6	7.88	K848
	N2	7/5	8.09	Embr. (2)		D2	2/3	8.57	Embr. (2?)	♀	U2	7/8	8.88	K841
♂	O1	7/12	7.14	E214	?♂	E1	2/12	7.69	Hatch	♀	V1	7/15	7.92	K902
	O2	7/14	8.15	Embr. (1½)	♂	E2	2/14	8.82	Embr. (5)	♀	V2	7/17	8.46	K884
♀	P1	7/21	6.62	E134	♀	F1	2/22	7.41	K387	♂	W1	7/24	7.10	K976
♀	P2	7/23	8.13	Broken	♀	F2	2/24	8.74	K332	♀	W2	7/26	8.47	K1000
♂	Q1	7/31	7.63	E288	♂	G1	3/4	7.78	Hatch					
♀	Q2	8/2	7.79	E286	♂	G2	3/6	8.62	Hatch					

Clutches 8 or 8 + days apart = 26♂ : 13♀ ; 7 da. = 9♂ : 23♀ ; 6 da. = 4♂ : 8♀
Sex unknown   9   8   0

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¹ The numbers opposite the abbreviation 'embr.' indicate the age attained by the embryo; those marked 15 days developed to full term, i.e., to the point of hatching (14 days = full term for second eggs since these are closely incubated from the beginning). 'Hatch' indicates hatching but dead before time to receive a tag or number (1 to 3 weeks). The rows of asterisks mark periods of reproductive rest.

TABLE 2

Record of ♀60—7/8 St. alba, 1/8 St. risoria.

SEX	CLUTCH	DATE	WEIGHT	NUMBER, OR REMARKS, OR YOLK WEIGHT
♀	A	4/24	7.59	1.575
♀	B1	5/1	7.74	1.460
♂	B2	5/3	8.79	1.840
♀	C1	5/15	7.50	Embr. (15)[1]
♂	C2	5/17	8.28	956
♀	D1	5/24	8.27	962
♀	D2	5/26	8.68	938
♀	E1	6/2	8.36	913
♀	E2	6/4	8.63	976
♀	F1	6/10	8.38	Trace dev.
	F2	6/12	9.13	Embr. (14)
♀	G1	6/19	8.48	907
♂	G2	6/21	9.00	921
?	H1	6/29	7.71	Embr. (15)
♂	H2	7/1	9.03	A291
♀	I1	7/7	7.57	Hatch
?	I2	7/9	8.75	Hatch

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SEX	CLUTCH	DATE	WEIGHT	NUMBER, OR REMARKS, OR YOLK WEIGHT
♂	S1	12/3	8.100	B299
♀	S2	12/5	7.725	B268
	T	12/15	8.430	Embr. (8)
♂	U1	12/28	7.800	B418
♂	U2	12/30	8.120	B401
♂	A1	1/9	7.915	A568
♀	A2	1/11	8.150	A552
♀	B	1/23	8.640	A559
♂	C1	2/3	8.400	A527
♂	C2	2/5	8.080	A508
♀	D1	2/14	8.230	A633
♀	D2	2/16	7.630	A561
♀	E1	2/25	8.680	A590
♂	E2	2/27	8.650	A573
♂	F1	3/5	8.065	A612
♀♀	F2	3/7	*10.080*	*Twins (14)*

SEX	CLUTCH	DATE	WEIGHT	NUMBER, OR REMARKS, OR YOLK WEIGHT
♀	O1	6/30	7.26	E154
♀	O2	7/2	7.53	Embr. (14)
♀	P1	7/12	6.72	E224
♀	P2	7/14	6.27	Embr. (14)
♂	Q1	7/31	7.42	E326
♀	Q2	8/2	7.48	E388
♂	R1	8/18	7.72	B499
♂	R2	8/20	7.90	B500
♀	S1	9/2	7.41	E356
♀	S2	9/4	7.62	E190
♀	T1	9/19	7.64	E549
♂	T2	9/21	7.95	E492
♂	U1	10/12	7.72	E577
♀	U2	10/14	8.45	E620
♂	V1	10/26	7.97	Hatch
♂	V2	10/28	8.03	E428
♀	W	12/3	8.23	E641

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Sex		Date	Number	Tag/status
♂	J1	7/18	8.79	A212
♂	J2	7/20	7.09	A252
♀	K1	7/26	7.89	Embr. (9)
	K2	7/28	8.79	A211
♀	L1	8/4	8.55	Hatch
?	L2	8/6	8.48	Embr. (10?)
♀	M1	8/13	8.09	B320
♂	M2	8/15	9.12	B322
♀	N1	8/24	8.47	B386
♀	N2	8/26	9.01	B363
?	O1	9/4	8.06	Hatch
♂	O2	9/6	8.66	Hatch
♀	P1	9/12	7.87	A352
♀	P2	9/14	8.90	A366
♀	Q	9/24	8.03	A369
♂	R1	11/19	8.290	B300
♀	R2	11/21	9.178	B295

Sex		Date	Number	Tag/status
♂	G1	3/13	7.700	Hatch
♀	G2	3/15	8.180	A668
♀	H1	4/28	7.08	A753
♂	H2	4/30	8.16	A720
♀	I1	5/6	7.93	A752
♂	I2	5/8	8.10	A755
♂	J1	5/15	7.60	A882
♀	J2	5/17	8.61	A890
♀	K1	5/23	7.61	B521
	K2	5/25	6.15	Infer.
♀	L1	6/2	8.15	A858
	L2	6/4	7.97	Embr. (5)
♂	M1	6/13	7.90	E76
♀	M2	6/15	8.38	E42
♀	N1	6/21	6.94	Hatch
	N2	6/23	7.90	E38

1917

Sex		Date	Number	Tag/status
♂	X1	12/20	8.29	B466
♀	X2	12/22	7.26	B474
♀	A1	1/7	7.73	E668
	A2	1/9	7.05	Embr. (2?)
♂	B1	1/22	7.74	Infer.
	B2	1/24	7.60	E681
♀	C1	2/10	7.69	K399
♀	C2	2/12	7.79	K388
♂	D1	3/2	7.90	Infer.
	D2	3/4	6.84	K408
♂	E1	3/23	8.15	K431
♀	E2	3/25	9.10	Embr. (14)
♂	F1	4/1	7.98	K453
♀	F2	4/3	8.11	K461

Clutches, 8 or 8 + days apart = 26♂ : 27♀ ; 7 days = 5♂ : 11♀ ; 6 days = 2♂ : 9♀
Sex unknown   8   2   5

¹ The numbers opposite the abbreviation 'embr.' indicate the age attained by the embryo; those marked 15 days developed to full term, i.e., to the point of hatching (14 days = full term for second eggs, since these are closely incubated from the beginning). 'Hatch' indicates hatching but dead before time to receive a tag or number (1 to 3 weeks). The row of asterisks mark periods of reproductive rest.

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TABLE 3

Further analysis of the data of table 2, ♀ No. A248 (nearly or quite pure St. risoria)

NUMBER	PERIODS OF REPRODUCTIVE ACTIVITY AND REST — Duration, kind and rate of reproductive period	CLUTCH INTERVALS OF PRECEDING COLUMN DIVIDED INTO FIRST AND SECOND HALVES; NUMBERS, ♂ AND ♀ AND SEX NOT KNOWN — Clutch interval	Ratio ♂ : ♀	Not incubated, not hatched, or sex unknown	AVERAGE EGG WEIGHT	WEIGHT OF FIRST AND SECOND EGGS OF CLUTCH, AND NUMBER ♂ : ♀ FROM EACH — Number	Weight	Ratio ♂	Ratio ♀	Sex unknown
		days				1st	7.14	5♂	: 5♀	(1)
1	Continuous activity	7.2	8 : 10	4	7.739	2nd	8.34	3♂	: 5♀	(3)
	Average days between (22) clutches = 7.4 days (17♂: 17♀)	7.6	9 : 7	6	7.575	1st	7.14	6♂	: 3♀	(3)
						2nd	8.00	4♂	: 4♀	(3)
	9/24/16—10/30/16					1st	7.39	1♂	: 1♀	(0)
2	Interrupted activity. Average days between (2) clutches = 16.5 days (2♂ : 1♀)	16.5	2 : 1	1	7.66	2nd	7.93	1♂	: 0♀	(1)
	12/1/16—4/9/17					1st	7.49	4♂	: 3♀	(0)
3	Continuous activity	8.1	7 : 4	4	8.06	2nd	8.64	3♂	: 1♀	(3)
	Average days between (15) clutches = 7.6 days (16♂ : 10♀)[1]	7.2	9 : 6[1]	2	8.34	1st	7.67	5♂	: 3♀	(0)
						2nd	9.01	4♂	: 3♀[1]	(1)
	4/16/17—8/3/17					1st	8.10	2♂	: 3♀	(1)
4	Continuous activity	7.0	2 : 8	2	8.52	2nd	8.94	0♂	: 5♀	(1)
	Average days between (12) clutches = 7.0 days (4♂ : 16♀)	7.0	2 : 8	2	8.26	1st	7.78	1♂	: 4♀	(1)
						2nd	8.73	1♂	: 4♀	(1)

Total from 1st of clutch = 24♂ : 22♀ (unknown, 4); 2nd = 16 ♂ :21♀ [2] (unknown 13).

[1] The last egg of this period produced female twins.

[2] There is an additional female twin. Reference to table 1 will show that there were 13 second eggs of the clutch, predominantly female-producing eggs, which were (in 12 cases) *too weak* in development to permit a test of sex. The sex of only 4 first eggs of the clutch is unknown; only 2 of the 4 were too weak to hatch. This undoubtedly makes both the apparent number (21) and the proportion of females from the second eggs of the clutch much too small.

TABLE 4

Further analysis of the data of table 1, ♀ No. 60 (hybrid 7/8 St. alba, 1/8 St. risoria)

NUMBER	PERIODS OF REPRODUCTIVE ACTIVITY AND REST — Duration, kind and rate of reproductive period	Clutch interval	Ratio ♂ : ♀	Not incubated, not hatched, or sex unknown	AVERAGE EGG WEIGHT	Number	Weight	Ratio ♂	Ratio ♀	Sex unknown
		days								
	4/24/15—9/24/15	7.7	3 : 7	5	8.372	1st	8.06	0♂	: 4♀	(2)
1	Continuous activity					2nd	8.79	3♂	: 3♀	(0)
	Average days between (16)	7.7	3 : 8	6	8.360	1st	8.16	1♂	: 4♀	(3)
	clutches = 7.7 days (6♂ : 15♀)					2nd	8.60	2♂	: 3♀	(3)
						Sing.	8.03	—	: 1♀	(0)
	11/19/15—3/15/16					1st	8.03	4♂	: 0♀	(0)
2	Continuous activity	11.4	5 : 4	1	8.235	2nd	8.44	1♂	: 3♀	(0)
						Sing.	8.55	—	: 1♀	(1)
	Average days between (10)[1]					1st	8.22	3♂	: 2♀	(0)
	clutches = 9.9 days (10♂ : 10♀)[2]	8.4	5 : 6[2]	0	8.370	2nd	8.52	3♂	: 4♀ [2]	(0)
	4/28/16—7/14/16	6.8	3 : 4	1	7.655	1st	7.55	1♂	: 3♀	(0)
3	Continuous activity					2nd	7.75	2♂	: 1♀	(1)
	Average days between (8)	7.4	1 : 7	2	7.502	1st	7.39	1♂	: 3♀	(1)
	clutches = 7.1 days (4♂ : 11♀)					2nd	7.61	0♂	: 4♀	(1)
	7/15/16—3/25/17					1st	7.78	7♂	: 3♀	(2)
4	Interrupted activity	14.5	12 : 10	3	7.820	2nd	7.78	5♂	: 7♀	(1)
	Average days between (13) clutches = 14.5 days (12♂ : 10♀)					Sing.	8.23	—	: 1♀	(0)

Total from 1st of clutch = 17♂ : 19♀ (unknown 8); 2nd = 16♂ : 25♀ (unknown 6).

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[1] Eleven clutches are present, but the first following a rest is left out of account in rating the time between clutches.

[2] Female twins were produced at the close of this period.

TABLE 5

Individual clutches of ♀ A248 which yielded the two sexes

SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE	SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE	SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE	SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE
♂	G1	7.07		♂	R1	7.63		♀	H1	7.81		♂	M1	8.22	
♀	G2	8.28	+17.1	♀	R2	7.79	+ 2.1	♂	H2	9.17	+17.4	♀	M2	8.70	+ 5.9
♂	K1	6.77		♀	Z1	7.56		♀	J1	7.18		♀	S1	8.01	
♀	K2	8.29	+22.4	?♂	Z2	8.48	+12.2	?♂	J2	8.51	+18.5	♂	S2	8.70	+ 8.6
♀	L1	6.99		♂	A1	7.28		♂	K1	7.43		♂	W1	7.10	
♂	L2	8.36	+19.6	♀	A2	8.47	+16.3	♀♀	K2	*10.63*	+43.1	♀	W2	8.47	+19.3

TABLE 6

Individual clutches of ♀ 60 which yielded the two sexes

SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE	SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE	SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE	SEX	CLUTCH	WEIGHT	PER CENT OF DIFFERENCE
♀	C1	7.50		♂	A1	7.915		♀	I1	7.93		♂	U1	7.72	
♂	C2	8.28	+10.4	♀	A2	8.150	+ 2.9	♂	I2	8.10	+ 2.1	♀	U2	8.45	+ 9.4
♀	G1	8.48		♀	E1	8.680		♂	J1	7.60		♂	X1	8.29	
♂	G2	9.00	+ 6.1	♂	E2	8.650	− 0.3	♀	J2	8.61	+13.3	♀	X2	7.26	−14.2
♀	M1	8.09		♂	F1	8.065		♂	M1	7.90		♂	C1	7.69	
♂	M2	9.12	+12.7	♀♀	F2	*10.080*	+24.9	♀	M2	8.38	+ 6.1	♀	C2	7.79	+ 1.3
♂	R1	8.290		♂	G1	7.700		♂	Q1	7.42		♂	E1	8.15	
♀	R2	9.178	+10.7	♀	G2	8.180	+ 6.2	♀	Q2	7.48	+ 0.8	♀	E2	9.10	+11.6
♂	S1	8.100		♀	H1	7.08		♀	T1	7.64					
♀	S2	7.727	− 4.8	♂	H2	8.16	+15.2	♂	T2	7.95	+ 4.0				

TABLE 7

Clutches (pairs of eggs) of greatest size disparity laid by Streptopelia risoria

61	♀ H1	6.96		Hatched		86	♀ B1	7.290		Hatched[1]	
	♀ H2	8.55	+22.8[2]	Hatched			♂ B2	8.870	+21.7[2]	Hatched	
	♂ K1	6.77		Hatched			♂ K1	7.150		Hatched	
	♀ K2	8.29	+22.4	Hatched			♂ K2	8.740	+22.2	Hatched	
	♀ Q1	6.62		Hatched			♀ M1	7.40		Hatched	
	Q2	8.13	+22.8	Broken			♀ M2	9.25	+25.0	Hatched	
	♂ K1	7.43		Hatched			♀ S1	7.300		Hatched	
	♀♀ K2	10.63	+43.1	*Twins*			S2	8.780	+20.3	Broken	
100	B1	8.270		1.685			♂ T1	6.980		Hatched	
	B2	9.970	+20.5	2.270	+34.7[2]		♀ T2	8.410	+20.5	Hatched	
Y	D1	7.550		1.480		141	E1	7.66		Hatched[1]	
	D2	9.265	+22.7	2.200	+48.7		♀ E2	9.22	+20.3	Hatched	
	F1	8.410		1.820			? ♂ F1	7.28		10-12 da. embr.	
	F2	10.445	+24.2	2.256	+23.9		♀ F2	8.86	+21.7	Hatched	
163	A1	5.145		0.943*		122	A1	7.010		1.550	
	A2	6.550	+27.3	1.170	+24.1		A2	8.730	+24.5	2.052	+32.4[2]
	♀ D1	7.47		Hatched			H1	7.420		1.600	
	♀ D2	9.04	+21.0	Hatched			H2	8.945	+20.5	2.035	+27.2
118	♂ X1	7.005		Hatched			G1	7.400		1.648	
	♀ X2	8.635	+23.2	13-14 da. embr.			G2	9.045	+22.2	2.132	+29.4

* Eggs thus marked are the first eggs in the life of the birds that produced them.

[1] The purity of the female parent is questionable.

[2] The figures of this column, in this table and in those which follow, represent the per cent of difference between the weights of the two eggs or of the two yolks.   In all cases the smaller egg or yolk is taken as 100 per cent.

TABLE 8

Clutches of eggs of greatest size disparity laid by hybrids of St. alba and St. risoria

ID	Clutch					ID	Clutch				
73	♂F1	8.065			Hatched	173	C1	8.800		2.010	
	♀♀F2	10.080	+24.9		Twins		C2	7.820	−12.5	1.485	−35.3
	♀K1	7.61			Hatched	311	E1	7.25		Infertile	
	K2	6.15	−23.7		Infertile		E2	8.72	+20.2	Infertile	
179a	B1	7.498		1.806		198	C1	7.900		1.625	
	B2	9.180	+22.4	2.038	+12.8		C2	9.425	+19.3	2.200	+35.5
101	D1	6.650		1.300		172	C1	6.620		0.990	
	D2	8.200	23.3	1.700	+30.8		C2	7.760	+17.2	1.435	+44.9
	C1	6.800		1.445		127c	A1	6.150			
	C2	8.520	+25.3	1.950	+34.9		A2	7.650	+24.4	0.930*	+45.2
177	J1	7.25		1.405		LL	F1	8.502		1.960	
	J2	9.33	+28.7	2.080	+48.0		F2	10.307	+21.2	2.932	+49.6
31	A1	5.590		1.100*		N2	J1	6.746		1.240	
	A2	6.750	+20.7	1.370	+24.5		J2	8.255	+22.4	1.725	+59.1
125k	A1	7.950		1.720*		OD	B1	7.855		1.818	
	A2	9.060	+13.9	2.080	+20.9		B2	8.630	+22.4	1.903	+ 4.7
PP	D1	8.375		1.827		201	C1	7.450		1.780	
	D2	7.270	−15.2	1.330	−37.3		C2	9.290	+24.7	2.350	+32.0
162	N1	6.53		1.170		271	D1	0.430		1.525	
	N2	7.86	+20.3	1.773	+51.5		D2	9.680	+14.8	2.080	+36.4
199	D1	7.615		1.400		272	A1	7.09		1.680*	
	D2	8.750	+14.9	1.910	+36.5		A2	8.53	+20.3	2.060	+22.6
281a	A1	6.973		1.601			B1	7.48		1.825	
	A2	8.082	+15.9	2.190	+36.8		B2	9.09	+21.5	2.250	+23.3
288a	B1	7.740		1.480		185	C1	7.140		1.610	
	B2	9.410	+21.5	2.050	+38.5		C2	8.660	+21.3	2.110	+31.0
	♂C1	7.915			Hatched	202	B1	8.80		2.246	
	♀C2	9.568	+20.9		Hatched		B2	7.21	−22.0	1.555	−44.4
135	A1	5.645		0.870*		200	F1	7.975		1.595	
	♀A2	6.845	+21.2		Hatched		F2	9.560	+19.8	2.205	+44.5

TABLE 8—Continued

153	B1	7.337		1.672			B1	8.325		1.970	
	B2	9.015	+22.8	1.818	+ 8.7		B2	9.890	+18.8	2.890	+46.6
188	E1	7.465		1.825			H1	7.515		1.750	
	E2	9.050	+21.2	2.055	+12.6		H2	9.018	+20.0	2.321	+32.6
153a	A1	6.850		0.750*		295a	A1	8.030		1.678	
	A2	8.190	+19.5	1.800	+140.0		A2	9.704	+20.8	2.135	+27.1
291	F1	7.140		1.550		156	D1	7.780		1.580	
	F2	8.470	+18.6	2.100	+35.5		D2	9.660	+24.1	1.975	+25.0
61b	♀E1	8.200		Hatched		159	P1	6.70		Infertile	
	♀E2	10.080	+22.9	Hatched			♀P2	8.04	+20.0	Hatched	
	♀X1	7.580		Hatched			♀G1	7.54		Hatched	
	♂X2	9.970	+31.5	Hatched			♀G2	9.36	+24.1	Hatched	
105	?♀B1	7.010		Hatched			L1	7.35		Broken	
	♂B2	8.590	+22.5	Hatched			♂L2	9.27	+26.1	Hatched	
	?♂C1	7.242		22 da. (!) live embr.			♀M1	7.26		Hatched	
	C2	8.800	+21.5	14 da. dead embr.			♂M2	8.97	+23.5	Hatched	
	M1	7.575		1.625		146	♂E1	7.69		Hatched	
	M2	9.340	+23.5	2.039	+25.5		♀E2	9.57	+24.4	Hatched	
	R1	7.630		1.575			B1	6.97		1.520	
	R2	9.690	+26.9	2.125	+34.9		B2	8.69	+24.7	1.810	+19.0
137	♂B1	7.140		Hatched		265	♀Q1	8.36		Hatched[1]	
	♂B2	8.700	+21.8	Hatched			♂Q2	10.31	+23.3	Hatched	

* Eggs thus marked are the very first eggs in the life of the birds that produced them.

[1] These eggs were the first after a long rest that followed a long peroid of reproductive overwork. It is also important to observe that they were fertilized by a generic hybrid (Zenaidura-Zenaida) male, and that these genera belong to a different subfamily from the female that produced these eggs. There is evidence that in this type of cross different sperms exercise opposite influence on the sex development.

It is of some interest to add that the male that developed from Q2 was killed when quite healthy; two testes were found, but, contrary to the rule for normal males, the left testis was larger than the right, i.e., the size relations of the glands were those of a female. (Riddle, Anat. Rec., vol. 14, 1918, pp. 283–334.)

TABLE 9

Clutches of greatest size disparity laid by St. alba and by Turtur orientalis

23	♀H1	7.210		Hatched		136	K1	7.14		Infertile	
	?H2	8.860	+22.9	Hatched			?♀K2	8.85	+23.9	Hatched	
139	L1	8.320		Lost			♂J1	7.510		Hatched	
	L2	10.042	+20.7	3 da. embr.			?♂J2	9.030	+20.2	Hatched	
155	R1	9.10		1.485		97	J1	7.210		1.276	
	R2	11.21	+23.2	2.486	+67.4		J2	8.920	+23.7	1.503	+17.8

TABLE 12

Size disparity of eggs of common pigeons

291a	B1	15.00		3.080		124	A1	17.365		3.928	
	B2	15.38	+ 2.5	1.760[1]	−75.0(?)		A2	14.065	−23.4	3.220	−21.
3 Ft.	B1	16.18		3.190		305	A1	10.50		0.995	
	B2	13.62	−18.8	2.350	−35.7		A2	11.65	+10.9	1.600	+60.8

[1] It is quite probable that this figure was copied wrong when the weighing was made, and should be 2.760.

TABLE 10

Clutches of greatest size disparity laid by miscellaneous hybrids (a few from pure species)

No.						No.					
140b	A1	6.618		1.543*		97	♂K1	7.98		Hatched	
	A2	8.357	+ 26.2	1.940	+25.7		♂K2	9.84	+23.3	Hatched	
127a	K1	5.39		1.095		196	♂E1	7.93		Hatched	
	K2	6.72	+ 24.7	1.160	+ 5.9		♀E2	10.03	+26.5	Hatched	
286	A1	6.34		1.170*		279	A1	5.41		1.080*	
	A2	8.35	+ 31.7	1.850	+58.1		A2	6.73	+24.4	1.540	+42.6
117	♀D1	7.28		Hatched		286	A1	6.34		1.170	
	D2	10.31	+ 41.6	2 da. embr.			A2	8.35	+31.7	1.850	+58.1
281	B1			0.840		174	I1	8.34		1.710	
	B2			0.450	−86.7		I2	5.28	−57.9	1.738	+ 1.6[1]
	G1			0.690		281	J1	5.10		1.075	
	G2			0.450	−53.3		J2	5.23	+ 2.5	1.480	+37.7
	L1	3.480[2]		0.550		273	♀A1	5.94		Hatched	
	L2	4.780	+ 37.3	0.965	+75.5		A2	7.25	+22.0	2-3 da. devel.	
	N1	4.20		0.785		5	P1	6.700		1.205	
	N2	1.96	−114.3	0.475	−65.3		P2	6.920	+ 3.3	1.783	+47.9
181	C1	3.985		0.628			E1	6.800		1.420	
	C2	3.380	− 17.9	0.448	−40.2		E2	5.650	−20.3	1.043	−36.1
54	D1	9.165		1.269		114a	L1	7.940		1.530	
	D2	10.120	+ 10.4	1.713	+35.0		L2	9.690	+22.0	1.640	+ 7.2

* Eggs thus marked are the very first eggs in the life of the birds that produced them.

[1] This second yolk absorbed water from the albumen during fifty-two hours, the first yolk for only five hours; if this correction were made the second yolk would be shown to be smaller than the first.

[2] Many eggs with imperfect shells or without shells in this series.

TABLE 11

Clutches of greatest size disparity laid by hybrids of St. alba and T. orientalis

3	E1	9.610		2.244		119	B1	10.345		2.840	
	E2	8.100	−18.6	1.485	− 51.1		B2	8.005	−29.2	1.620	−75.3
98	L1	8.600		1.050¹		100	L1	10.050		2.155	
	L2	6.970	−23.4	1.560	+48.6 (?)		L2	8.360	−20.2	1.870	−15.2
49	H1	9.535		1.992		109	J1	8.575		1.430	
	H2	8.270	−15.3	1.370	− 45.4		J2	10.200	+18.9	2.220	+55.2
	E1	8.620		1.534			B1	9.310		2.080	
	E2	9.800	+13.7	2.220	+ 44.7		B2	11.500	+23.5	3.020	+45.2
103	H1	8.915		2.057		89	D1	7.27		1.190	
	H2	6.730	−32.4	0.975	−110.97		D2	8.27	+13.7	1.650	+38.6
258	G1	7.730		1.450			E1	8.76		1.830	
	G2	9.590	+24.0	2.440	+ 68.3		E2	6.66	−31.5	1.100	−66.3
33	T1	7.260		1.615		65	A1	7.245		1.498	
	T2	9.120	+25.5	2.110	+ 30.6		A2	8.650	+19.4	2.033	+35.7
49	L1	9.700		2.088			D1	7.505		1.250	
	L2	7.360	−31.8	1.480	− 41.1		D2	8.840	+17.8	1.960	+56.8
106	G1	8.75		1.510			I1	8.870		2.070	
	G2	9.94	+13.6	2.162	+ 43.2		I2	7.210	−23.1	1.230	−68.3
	C1	8.615		1.466			K1	7.670		1.380	
	C2	9.470	+ 9.9	2.080	+ 41.9		K2	9.250	+20.6	2.001	+45.0
115	D1	8.370		1.905			F1	7.585		1.355	
	D2	6.017	−39.1	0.915	−108.2		F2	9.035	+19.1	1.906	+40.7
30	B1	8.455		1.602		56a	J1	8.670		1.890	
	B2	9.800	+15.9	2.200	+ 37.3		J2	6.505	−33.2	1.220	−54.9
	I1	7.755		1.345		29	G1	9.520		2.100	
	I2	9.605	+23.8	2.115	+ 57.2		G2	8.070	−17.9	1.520	−38.2
16	C1	10.030		2.530			W1	8.100		1.483	
	C2	8.600	−16.6	1.810	− 39.8		W2	9.340	+15.3	2.015	+35.9

¹ It is probable that this weight was 2.050 instead of 1.050 as recorded. The usual 'underscoring' of yolks of abnormal size for the bird or species was here omitted at the time of weighing.

TABLE 11—Continued

14	E1	8.120		1.295		110	H1	8.360		1.525	
	E2	8.350	+ 2.8	1.845	+ 42.5		H2	9.255	+10.7	2.315	+51.8
112	C1	6.970		1.150			F1	9.320		1.925	
	C2	8.870	+27.2	1.970	+ 71.3		F2	6.990	−33.3	1.630	−18.1
	L1	8.367		2.120		206	♀Z1	6.04		Incubated	
	L2	6.795	−23.1	1.700	+ 24.7		♂Z2	7.31	+21.0	Incubated	
	B1	8.062		1.940		264	A1	9.110		1.520	
	B2	6.760	−19.2	1.325	− 46.4		A2	9.850	+ 8.1	2.085	+37.2
152	A1	11.220		3.035		280	G1	6.73		1.290	
	A2	8.150	−37.6	1.610	− 88.5		G2	5.78	−16.4	0.850	− 51.8
	C1	8.340		1.855			H1	7.34		1.150	
	C2	7.050	−18.3	1.147	− 61.7		H2	5.76	−27.4	0.930	− 23.7
	H1	8.180		1.925			K1	5.57		0.774	
	H2	7.095	−15.3	1.240	− 55.2		K2	6.57	+17.9	1.268	+ 63.8
	E1	8.950		2.130			D1	5.27		0.645	
	E2	5.030	−77.9	0.495	−330.3		D2	6.82	+29.3	1.320	+104.6
	F1	7.270		1.400		285	L1	9.830		2.335	
	F2	9.180	+26.2	2.280	+ 62.8		L2	8.600	−14.3	1.630	− 43.3
	H1	7.940		1.870			H1	7.440		1.235	
	H2	7.240	− 9.6	1.370	− 36.5		H2	9.180	+23.4	2.160	+ 74.9
	I1	7.990		1.820			D1	10.098		2.502	
	I2	10.010	+25.3	2.610	+ 43.4		D2	8.677	−16.3	1.835	− 36.3
9	F1	9.410		2.025		18	A1	8.620		1.625	
	F2	7.500	−25.4	1.430	− 41.6		A2	6.300	−36.8	0.620	−162.1
	G1	7.625		1.267			B1	9.075		1.840	
	G2	9.155	+20.1	2.315	+ 82.7		B2	7.340	−23.6	1.580	− 16.5
	K1	9.750		1.873		104	G1	9.560		1.968	
	K2	7.130	−36.7	0.910	−105.8		G2	7.817	−22.3	1.665	− 18.2
	Y1	9.320		2.213		158	C1	9.590		1.920	
	Y2	7.440	−25.3	1.443	− 53.4		C2	7.680	−24.9	1.350	− 42.2
6	Y1	6.640		1.573		18	C1	8.680		2.080	
	Y2	8.200	+23.5	1.760	+ 11.9		C2	7.550	−14.9	1.472	− 43.3
49	B1	8.290		1.770		46	I1	6.940		1.205	
	B2	7.440	−11.4	1.310	− 35.1		I2	5.570	−24.6	0.782	− 54.1

TABLE 13

Weights of five eggs known to be 'double-yolked' compared with weights of eggs and yolks of other eggs of the clutch and with adjoining clutches

♀0 (prob. pure Stig. senegalensis), 1913

| R Wt = 6.700.........1.500 | S Wt. = 8.015 + 19.6 $\left\{\begin{array}{l}1.170\\1.122\end{array}\right.$ | A Wt. = 4.085[1] = 1.120 (+ shell) |

♀154 (hybr. alba × risoria), 1915

| A1 Wt. = 5.180........0.625 | B1 Wt. = 7.308 $\left\{\begin{array}{l}0.450\\0.833\end{array}\right.$ | C1 Wt. = 6.620 = 0.990 |
| A2 Wt. = 5.200........0.640 | B2 Wt. = 5.615 − 30.2   0.735 − 74.4 | C2 Wt. = 7.760 = 1.435 |

Not sure the vitelline membrane completely separated the yolks in B1

♀870 (hybr. orientalis × alba), 1916

| H1 Wt. = 8.910........1.930 | I1 Wt. = 9.150 1.812 | J1 Wt. = 9.600 = 2.150 |
| H2 Wt. = 8.635........2.240 | I2 Wt. = 10.270 + 12 2.360 + 30.2[2] | J2 Wt. = 9.170 = 2.150 |

♀TSA18 (hobr. senegalensis × ala), 1913

| N1 Wt. = 6.610........1.113 | O1 Wt. = 9.480 $\left\{\begin{array}{l}1.112\\1.100\end{array}\right.$ | P1 Wt. = 6.700 = 1.205 |
| N2 Wt. = 7.470........1.377 | O2 Wt = 7.278 − 30.3 1.300 − 70.1 | P2 Wt. = 6.920 = 1.783 |

♀189 (hybr. orient. − alba × alba − risoria), 1916

| G1 Wt. = 8.300........1.350 | H1 Wt. = 11.00 2.560[2] | I1 Wt. = 8.080 = 1.295 |
| G2 Wt. = 8.62.........1.400 | H2 Wt. = 8.65 − 27.1 1.610 − 59.0 | I2 Wt. = 8.500 = 1.405 |

[1] This egg was without a shell; its weight as given is therefore considerably too low.
[2] The two yolks were not separately weighed.

THE EFFECTS OF THE DUCTLESS GLANDS ON THE DEVELOPMENT OF THE FLESH FLIES

B. W. KUNKEL

From the Department of Biology, Lafayette College, Easton, Pa.

The effects on embryonic development of the ductless glands have not as yet been very widely studied, and our knowledge of their action is extremely meager. As practically all the experiments on the developing embryo have been made on vertebrates, it seemed desirable to determine if possible how some of the invertebrates react to these glands in which no organ comparable to a ductless gland has, so far as I know, been demonstrated.

In addition to the work of Gudernatsch on frogs and rats, recently Northrop has experimented on Drosophila and found that although the larvae of this fly do not develop normally on a sterile paste made by grinding fresh thyroid and thymus with glass, the larvae develop quite normally when bacteria are present in the cultures. Aside from these studies just mentioned, the only other animals which have been used have been Paramecium by Nowikoff, Shumway, and Budington and Harvey who also used Stylonichia.

In view of the suppression of growth and acceleration of differentiation which has been observed in the tadpole by Gudernatsch, it seemed especially desirable to test the effects of thyroid gland on an animal in which the processes of growth and differentiation are more or less completely separated, as is the case in the insects which have a complete metamorphosis. The flesh flies seemed to lend themselves to this purpose most perfectly because of their abundance and the ease of rearing them as well as because the larvae consume the ductless glands with avidity and thrive on them.

The flesh flies which I found visiting exposed meat during the summer months are: Lucilia caesar, L. sericata, Calliphora erythrocephala, and a species of Sarcophaga, probably sarracena.

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Lucilia, as a rule, lays its eggs on meat before decay has set in, within a few minutes after the animal has been killed, while Sarcophaga is attracted to meat which has advanced somewhat in the process of decay.   Most of the observations made in this investigation were on Lucilia which is the most abundant genus of flesh flies in this region during the period of these observations. The eggs of Lucilia are laid in masses of about one hundred.   In a large number of observations I found that the flies prefer crevices or cavities in which to deposit their eggs, at least these situations are the first to receive eggs, later the more exposed portions become 'blown' as well.

As flies of different species sometimes lay their eggs in close contact with each other so that the limits of the two or more masses cannot be distinguished, care was taken to use only isolated egg masses of comparatively small size which were laid soon after the meat was exposed to the flies.

The eggs of Lucilia hatch in about twenty-four hours and the larvae at once begin to liquefy the food material.   They work beneath the surface, excavating cavities into which they sink. Finally the whole mass of food may be reduced to a thick, gray broth through which the larvae move freely.   The larvae are very strongly negatively phototactic and are apparently very sensitive to variations in temperature and in the composition of the atmosphere.   If for any reason, such as improper ventilation, the conditions in the culture glass in which the larvae are feeding become unfavorable, within less than a minute there is a whole-sale movement on their part to escape.

L. caesar and sericata are very closely related species which are distinguished by the arrangement of the spines on the thorax. I am indebted to Mr. V. A. E. Daecke of the Pennsylvania State Agricultural Department at Harrisburg for the identification of the flesh flies.   Lucilia may be distinguished by the bright metallic luster of the body, which is green.   Calliphora has a black thorax and an abdomen which is a deep metallic blue. The species of Sarcophaga are grayish, and can usually be distinguished by the checkerboard pattern of gray and black squares on the abdomen.

The method of procedure in the experiments made was very simple. The food material with the eggs on it was placed in ordinary glass tumblers having a capacity of about 200 cc. which were covered with voile fastened with a rubber band. By examining the glasses several times a day any eggs deposited on the voile may be removed before they hatch and contaminate the culture. In later experiments a canopy of mosquito netting was stretched over the glasses to prevent the near approach of flies. When it was desired to obtain the pupae, it is more convenient to place the food in a Petri dish and place this upon a layer of sand 1 or 2 cm. deep in the bottom of a large culture glass 200 cm. in diameter. This large dish was covered with a glass plate elevated at one side by a few folds of paper for ventilation, and the whole was covered with a mosquito net and kept shielded from direct sun light. The culture dishes were always kept side by side under as nearly identical conditions of illumination and temperature as possible and in order to reduce any accidental differences, the positions of the dishes were reversed from time to time. As far as possible, in each observation a single egg mass was employed, one portion was used for the experimental feeding and the other for the control.

It is somewhat difficult to handle the eggs of the flesh flies without injury because of their stickiness. In consequence it frequently occurred that the division of the egg mass was far from equal.

The glands used for food were obtained fresh from the slaughter house and for the most part were obtained from calves, although some were obtained from steers and sheep. The control food was a mass of muscle approximately equal in bulk to the thyroid or thymus used. An advantage in using these relatively small masses of food is that the heating due to active putrefaction is less.

The effect of the ductless glands on growth and differentiation was measured by the length of the larvae, the length of the pupae, and the duration of the larval and pupal stages.

In order to measure the larvae, they were killed by immersion in boiling water or 95 per cent alcohol. Both these agents seemed to cause very little contraction or distortion. It was

found difficult to measure the length of the larvae very accu-
rately because of their consistency and the protrusion of the
mouth parts. The length was obtained with dividers and a milli-
meter scale. It was soon found, however, that sufficient accu-
racy was not possible by this means, and so in later experiments
the length of the pupae was used as an index of the effect of special
feeding on the growth of the larvae. By means of micrometer
calipers it was possible to measure the pupae to .001 inch.

A number of observations was made to determine the effect
of thyroid feeding on the length of the larvae. From these, the
following have been selected because of the completeness of the
data regarding the flies. In all the observations on the length
of the larvae there was an abundance of food present so that
effects are not due to insufficient food. Unfortunately, I did not
identify the adults, but judging from the characters of the larvae,
there is no doubt but that we are dealing with Lucilia.

Measurements were made of larvae varying in age from two to
eight days and in all these cases except in the youngest larvae of
two days' age, the muscle-fed larvae were slightly longer than the
thyroid-fed. Except in the eight-day larvae, the difference in
length is rather insignificant. Great care was taken in the meas-
urement of the larvae not to estimate the thyroid larvae low and
the muscle larvae high.

The results are shown in the following table:

TABLE 1

*Showing the effect of thyroid feeding on the average length of larvae of
different ages*

Age of larvae in days	2	3	6	7	8
Egg mass number	15	17	18	5	4
Average length in mm. thyroid-fed	13	12.4	15.6	10.9	13.4
Average length in mm. muscle-fed	12.6	12.6	15.9	11.4	14.9

Table 2 indicates the distribution of the larvae in egg mass No.
5. In this experiment, after seven days of feeding twenty larvae
from the thyroid and twenty from the muscle, chosen at random,
were measured after killing with boiling water.

TABLE 2

Showing the effect on the length of larvae of feeding thyroid exclusively. The first line indicates the distribution of the thyroid-fed larvae according to length, the second line indicates the distribution of the controls which were fed on muscle

Thyroid	1	1	5	0	9	3	1
Muscle	0	0	1	3	6	5	5
Length in mm	9.5–9.9	10–10.4	10.5–10.9	11–11.4	11.5–11.9	12–12.4	12.5–12.9

In order to compare the effects of thyroid and thymus feeding on the length of the larvae, reference may be made to egg mass No. 20. This was a very large egg mass numbering nearly 200. Portions of the eggs were placed on thyroid and thymus of a calf, respectively. At the end of four days the larvae were killed with boiling water and measured. The average length of 107 thyroid larvae was 11 mm. and that of 71 thymus larvae was 14 mm. (table 3).

TABLE 3

Showing the effect on the length of larvae of feeding thyroid and thymus, respectively. The first line indicates the distribution of the thyroid-fed larvae according to length, the second line indicates that of the thymus-fed larvae

Thyroid	6	34	29	6	4	12	13	3	0	0
Thymus	0	0	0	4	17	15	12	15	6	2
Length in mm	8–8.9	9–9.9	10–10.9	11–11.9	12–12.9	13–13.9	14–14.9	15–15.9	16–16.9	17–17.9

The effect of thyroid feeding on the duration of larval life could not be determined with great accuracy, as pupation is retarded if the larvae are prevented from burying themselves or if they are disturbed after burial. The length of time during which the larvae feed is not affected markedly by the ductless glands selected for the experiment. This is rather interesting in view of the fact that the pupation of mature larvae of Lucilia caesar may be retarded in several ways; such as by cutting off the supply of oxygen, as Dewitz ('01) has shown. I have noticed also in my own experiments that if the substratum is too dry the pupation of mature larvae may be delayed from forty-eight hours, which is normal, to four or five days.

Nearly all my evidence indicates that there is some hastening of maturity effected by feeding thyroid gland to larvae of the

flesh flies, although the acceleration is not great.   In one excep-
tional case, that of egg mass No. 28 of L. caesar there was a slight
retardation in pupating effected by thyroid feeding.   My record
in this experiment shows that on the sixth day after deposition of
eggs, the muscle larvae were all buried while the thyroid larvae
were in process of burial.   The following day 8 per cent of the
thyroid larvae had pupated and 16 per cent of the muscle larvae.
On the other hand, eighteen days after the deposition of the eggs,
the thyroid-fed had outstripped the muscle-fed, for 57 per cent
of the former had emerged and only 18 per cent of the latter.

Egg mass No. 16 of L. caesar, in which the thyroid-fed larvae
were markedly smaller than the muscle-fed affords evidence that
thyroid feeding accelerates development.   Ten days after depo-
sition of eggs, the thyroid culture contained 94 pupae and 1
larva, while the muscle culture contained only 43 pupae and 11
larvae.   That is, 99 per cent of the larvae had pupated in the
thyroid and only 80 per cent in the muscle culture.

Egg mass No. 9 also indicates that the pupal period may be
shortened slightly by thyroid feeding.   In both experimental
and control cultures pupation was first noted on the tenth day
following the deposition of the eggs.   On the fourteenth day,
five out of fourteen pupae had opened while none of the muscle
pupae had matured.   Within eighteen days after the deposition
of the eggs all ten flies from the muscle-fee larvae had emerged,
but the rest of the thyroid-fed were dead.

Egg mass No. 16 also affords some evidence that the pupation
period may be slightly shortened by thyroid feeding.   Fourteen
days after deposition of eggs, 43 out of 94, 46 per cent, of the
thyroid pupae had emerged while in the same time only 2 out of
50, 4 per cent, of the muscle pupae had emerged.   Two days
later there were 36 muscle flies emerged and 88 thyroid flies, or
72 per cent of the muscle-fed flies had emerged in the same time
that 93 per cent of the thyroid-fed had.

Egg mass No. 22 of L. sericata also shows that there is a slight
hastening of pupation effected by thyroid feeding.   Six days
after egg deposition there were found in the muscle culture
5 pupae and 57 larvae which had buried themselves, while in the

thyroid culture there were 46 pupae and 29 larvae.   That is, less
than 9 per cent of the muscle larvae had pupated in the time in
which over 60 per cent of the thyroid-fed had pupated.   Eleven
days after deposition there were 54 pupae in the muscle culture
and 66 in the thyroid.

The experiments to determine the effects of thyroid feeding on
the size of the pupae were on the whole rather disconcerting,
for although accurate measurements were possible there were less
constant and definite differences from the controls than in the
experiments with the larvae.   In two experiments with L.
sericata the pupae derived from muscle-fed larvae were markedly
longer than those derived from thyroid-fed.   A similar difference
was noted also in one of the experiments with L. caesar.   In
two experiments to compare the effects of thyroid and thymus
feeding, there was a very slight superiority in the length of the
thyroid-fed pupae.   In two experiments, on the other hand, in
which egg masses were placed on muscle and thymus, respectively,
there was a superiority in length among the thymus-fed.   It is
evident that the results are not striking and that the effect of
thymus and thyroid probably are to a slight extent in accord with
the findings of others who have worked with tadpoles.

Egg mass No. 13 of L. sericata is typical of a number of experi-
ments.   Larvae were fed exclusively on calf thyroid and muscle.
After ten days all the larvae in both cultures had pupated.   The
distribution of the pupae is indicated in the following table
(table 4):

TABLE 4

*Showing the effect on the length of pupae of Lucilia sericata of feeding the larvae
exclusively on thyroid.   The first line indicates the distribution of the thyroid-fed
pupae according to length, the second line that of the muscle-fed*

Thyroid...	2	4	2	3	7	5	10	4	3
Muscle....	0	0	4	8	5	4	6	5	3
Length in .001's inch.....	270–274	275–279	280–284	285–289	290–294	295–299	300–304	305–309	310–314

The average length of the thyroid pupae was .295 inch; that
of the muscle pupae was .296.   This difference, of course, is too
small to be significant.

The pupae were then removed from the sand and placed in Petri dishes with loose-fitting covers. After seven days all the living pupae had emerged. There seemed to be greater mortality among the thyroid fed flies than among the muscle-fed, for out of 35 muscle pupae there emerged 33 adults, while from the 40 thyroid pupae only 23 emerged.

In egg mass No. 24 of L. caesar, the thyroid-fed larvae produced pupae having an average length of .315 inch, and the muscle-fed larvae produced pupae having a length of .314 inch. In egg mass No. 26 of L. caesar thyroid feeding was followed by a slightly decreased length of pupae in comparison with those fed on muscle. The respective average lengths were .293 and .297 inch (table 5).

TABLE 5

Showing the effect on the length of the pupae of Lucilia caesar of feeding the larvae on thyroid. The first line indicates the distribution of the thyroid-fed pupae according to length, the second line indicates that of the muscle-fed

Thyroid	1	5	6	1	4	1	1
Muscle	0	1	3	2	3	4	0
Length in .001's inch	280–284	285–289	290–294	295–299	300–304	305–309	310–314

Egg mass No. 16 of L. caesar shows by far the greatest difference in length between the thyroid- and muscle-fed pupae. The distribution of the pupae is shown in table 6. The average length of the muscle-fed pupae was .350 inch, and that of the thyroid-fed was .308 inch.

TABLE 6

Showing the effect on the length of the pupae of Lucilia caesar of feeding the larvae on thyroid. The first line indicates the distribution according to length of the thyroid-fed pupae, the second indicates that of the muscle-fed

Thyroid	3	0	0	1	3	15	17	15	11	11	13	4	0	0	0	0	1	0	0
Muscle	0	0	0	0	0	0	0	0	0	0	2	2	2	9	7	14	10	3	1
Length in 0.001's inch.	270–274	275–279	280–284	285–289	290–294	295–299	300–304	305–309	310–314	315–319	320–324	325–329	330–334	335–339	340–344	345–349	350–354	355–359	360–364

In the case of egg mass No. 22 of L. sericata the food material was raised to the boiling-point before the eggs were placed on it. The average length of the thyroid pupae was .291 inch; that of the muscle pupae, .303 inch. The distribution of the pupae is shown in table 7.

TABLE 7

Showing the effect on the length of the pupae of Lucilia sericata of feeding the larvae on thyroid. The first line indicates the distribution according to length of the thyroid-fed pupae, the second indicates that of the muscle-fed

Thyroid	1	2	4	10	15	15	4	4	7	3	1	0	0
Muscle	0	0	0	0	4	10	8	8	6	8	7	2	1
Length in .001's inch	265–269	270–274	275–279	280–284	285–289	290–294	295–299	300–304	305–309	310–314	315–319	320–324	325–329

In a number of experiment the effects of thyroid and thymus feeding were compared. Egg masses No. 24 and No. 26 show that thymus probably has an effect upon the size of the pupae. In one lot in whi ch there were 25 muscle pupae and 52 thymus pupae the average respective lengths were .314 and .322 inch (table 8).

TABLE 8

Showing the effect on the length of the pupae of Lucilia caesar of feeding the larvae on thymus. The first line indicates the distribution according to length of the thymus-fed pupae, the second line indicates that of the muscle-fed

Thymus	0	0	1	4	9	9	9	6	6	3	5
Muscle	1	2	1	4	3	6	4	3	1	0	0
Length in .001's inch	290–294	295–299	300–304	305–309	310–314	315–319	320–324	325–329	330–334	335–339	340–344

In another series, however, in which there were 15 muscle pupae and 13 thymus pupae, the respective lengths were .297 and .298 inch (table 9).

TABLE 9

Showing the effect on the length of the pupae of Lucilia caesar of feeding the larvae on thymus The first line indicates the distribution of the thymus-fed pupae according to length, the second line indicates that of the muscle-fed pupae

Thymus	2	1	4	3	3	1	1
Muscle	0	1	3	2	3	4	0
Length in .001's inch	280–284	285–289	290–294	295–299	300–304	305–309	310–314

In summing up the results of these experiments, it may be said
that they agree essentially with those already determined for
vertebrates, but are far less striking.   Feeding larvae of Lucilia
exclusively upon mammalian thyroid tends to retard slightly   the
growth of the larvae and consequently to reduce the size of the
resulting pupae, while thymus tends to increase their size.
Thyroid feeding tends to hasten the onset of pupation and to
shorten the period of pupation.

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IS THE THEORY OF AXIAL GRADIENT IN THE REGENERATION OF TUBULARIA SUPPORTED BY FACTS?

MARIO GARCIA-BANUS

From the Laboratories of the Rockefeller Institute for Medical Research

In his book "Individuality in Organisms," C. M. Child assumes the existence of 'metabolic gradients' in a great number of species of animals and plants, and on this assumption he builds a theory of individuality.

In the case of the hydroid Tubularia, which he uses extensively to prove his theory, the metabolic gradient would lie in the axis of the animal.

The apical end of the metabolic gradient of the major axis is the apical end of the hydranth and from there the rate (metabolic rate) decreases basally through the hydranth. In the stem the metabolic rate is lower than in the hydranth and there is a slight decrease in the basal direction, but at the growing tip of the stolon there is a short gradient in the opposite direction.[1]

Child has made no measurements of the rate of metabolism of different regions of the stem of tubularia. What he really means is that an excised piece of the stem of Tubularia regenerates a new hydranth at the oral end the more rapidly, the nearer this end lies to the original apex of the stem. Such differences he assumes to be due to alleged differences in what he calls 'metabolic rates.' We are therefore only concerned with the question whether the regional differences in the rate of regeneration which Child assumes in Tubularia really exist.

If a piece is cut from a stem of Tubularia a new hydranth will regenerate at each end, and, according to the old experiments of Loeb, the oral end of the piece will, as a rule, regenerate the

[1] Child, C. M., Individuality in organisms. Chicago, 1915.

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hydranth sooner than the aboral end. Child in his experiments[2] cut the stems of Tubularia in two or three pieces of equal length, and states that in such cases the oral end of the most apical piece will regenerate a hydranth sooner than the oral end of the most basal piece. This is the actual basis for his theory of 'axial gradient.'

The differences found by Child between the times of emergence of the oral hydranths of the two pieces are, however, so slight that the suspicion arises that they may be merely within the limits of error and individual variation.

As a matter of fact, only in one single case of those that he presents, is there a marked difference between the time of the regeneration of the oral hydranths of the apical and basal pieces. And in this case the average is based on the observation of only eight individual stems. The apical pieces give an average time of emergence of the oral hydranths equal to 27 hours, while for the basal pieces the average is 36.5 hours. In all the other cases which he gives, the differences are far from being so marked. Thus in one case the averages for the hydranth formation of eight stems were 38 hours for the apical pieces and 39 hours for the basal pieces, while in a third case the averages were 42 for the apical pieces and the same number 42 for the basal ones! In the case of the stem cut into three pieces of equal length the averages for the oral hydranth formation are for one of the series of twenty stems, 42.5 for the most apical piece and 44 for the most basal one, and in another series of ten stems the averages are 117 for the apical piece, 118 for the middle piece, and 120 for the most basal one.

The differences in all these experiments, except the first, are so small that they may well lie within the limits of variation and of error of such experiments. In order to be sure we repeated Child's experiments to try to ascertain the foundation for his conclusions.

[2] Child, C. M., An analysis of form-regulation in Tubularia. IV. Regional and polar differences in the time of the hydranth formation as a special case of regulation in a complex system. *Arch. f. Entwicklungsmech.*, 1907, 24, 1.

TABLE 1

Time in hours required for emergence of the oral hydrants of two pieces of equal length

NUMBER OF INDIVIDUAL	APICAL PIECE	BASAL PIECE
1	75	71
2	81	73
3	71	77
4	71	71
5	77	77
6	71	71
7	95	71
8	95	79
9	81	71
10	71	71
11	71	˙73
12	71	—
13	79	73
14	83	95
15	71	73
16	95	71
17	77	95
18	75	79
19	71	83
20	71	73
Averages	77.6 hours	76.1 hours

The experiments were carried out at New York (with material from Long Island Sound and from the Lower Bay) and at Woods Hole, and the results were similar in both places. The material and conditions, especially at Woods Hole, were so excellent that we were able to keep the regenerating stems alive in finger bowls for more than fifteen days.

In each case we recorded the time of emergence of the hydranth outside the perisarc, and observations were made at least every two hours.

In the first group of experiments (table 1), the stems were cut into two pieces of equal length; several series of this type, with twenty stems each were made with similar results, namely, that *there is no marked difference between the times of appearance of the oral hydranths of the two pieces.* Very often the oral hydranth of the basal piece is the first to appear. As an example of

this may serve the experiment recorded in table 1. The size of both pieces was 20 mm., so that they were long enough to show a marked difference according to Child's opinion. Of the twenty stems included in this experiment, in eight of them the oral hydranth of the apical piece appeared first, but there were seven cases in which the oral hydranth of the basal piece was the first to appear and four cases in which they both appeared at the same time. The averages of the time of appearance are 77.6 hours for the oral hydranths of the apical pieces and 76.1 hours for the oral hydranths of the basal pieces. The difference is even slightly in favor of the oral hydranth of the basal piece, but it is so small that it is within the limits of error or of individual variation.

In a second group of experiments the stems were cut into three pieces of equal length. The pieces in this case were each 10 mm. long and the results are similar to those of the other series.

The experiment in table 2 may serve as an example. There are in this case ten stems in which the oral hydranth of the third and most basal pieces emerges before the corresponding hydranths of the most apical pieces, while there are only five stems in which the oral hydranth of the most apical piece is the first to appear and there are two cases in which both appear at the same time. If we compare the apical piece with the middle piece, the results are the same; in seven cases out of the twenty, the oral hydranth of the middle piece is the first to appear, while there are only two cases in which the contrary happens, and six cases in which both appear at the same time.

The averages in this experiment are: 78.6 hours for the oral hydranths of the apical piece, 77.8 for those of the middle piece, and 74.2 for those of the basal pieces. This shows again that the slight difference in the time of appearance turns out to be in the opposite sense of what we ought to expect from Child's theories. We have made several series of this type with similar results, and in some cases the differences in favor of the basal pieces were even greater than in the case just shown. For instance, in one of these experiments in which twenty stems were cut into three pieces of 10 mm. each, the averages for the time of emergence of

TABLE 2

Time in hours required for the emergence of the oral hydranths of three pieces of equal length, from the same stem of Tubularia

Length of the pieces = 10 mm.

NUMBER OF INDIVIDUAL	APICAL PIECE	MIDDLE PIECE	BASAL PIECE
1	91	69	67
2	77	71	69
3	71	77	73
4	69	91	71
5	67	69	67
6	69	66	67
7	71	71	73
8	73	73	71
9	—	—	79
10	79	69	73
11	75	75	77
12	79	73	73
13	73	73	75
14	—	91	91
15	91	91	79
16	—	95	71
17	—	91	69
18	91	73	73
19	91	91	75
20	91	69	91
Averages..........	78.6 hours	77.8 hours	74.2 hours

the oral hydranths were in hours: for the apical piece 92, for the middle piece 84, and for the basal piece 76. We have chosen the experiment, the results of which are given in table 2, as an example, because in it the individual differences are not very great, so that the data are more constant and the averages more reliable. It is therefore a mere matter of chance whether the oral hydranths of the apical or basal pieces emerge first, as long as both pieces have equal length.

Comparing pieces of different size from the same stem, Child found that the shorter pieces will form hydranths a little later than the longer ones, but he states that since the more basal pieces will form the oral hydranths later than the more apical, the pieces must be cut in such a way that the shorter piece is always the more apical one. Thus he says the two factors of size and level are opposed to each other instead of acting in the same

TABLE 3

Time in hours required for emergence of the oral hydranths in pieces of different length

Ratio of the length of the pieces = 1 : 2.
Series A   Apical piece = 10 mm.   Basal piece = 20 mm.
Series B   Apical piece = 20 mm.   Basal piece = 10 mm.

	SERIES A			SERIES B	
Number of individual	Apical piece 10 mm.	Basal piece 20 mm.	Number of individual	Apical piece 20 mm.	Basal piece 10 mm.
1	67	67	1	67	67
2	53	53	2	67	67
3	55	55	3	53	69
4	55	67	4	55	69
5	55	55	5	67	97
6	53	55	6	—	67
7	67	67	7	79	97
8	—	67	8	91	67
9	67	67	9	91	71
10	73	67	10	67	67
11	75	67	11	59	71
12	67	55	12	91	91
13	55	67	13	59	67
14	71	67	14	67	67
15	69	67	15	67	67
16	91	67	16	67	67
17	˙95	73	17	67	67
18	53	66	18	77	67
19	67	67	19	67	67
20	55	67	20	53	67
Averages.......	65.4	64.2		69.0	70.3
Differences ....1.2 hours					1.3 hours

sense and adding their effects, as would be the case if the shorter piece were basal to the larger one.

We have found the existence of this factor of the size of the pieces to act constantly but irregularly, and we have tested the other factor of level comparing a series of stems cut in such a way that the shorter piece is the more apical with another series of stems, under the same conditions and cut into the same proportions, but in which the shorter piece is the more basal one.

The results are given in tables 3 to 5. In the experiment recorded in table 3 the ratio of the lengths of the pieces was 1 : 2.

TABLE 4

Time in hours required for emergence of the oral hydranths in pieces of different length

Ratio of the length of the pieces = 1 : 3.
Series A   Apical piece = 10 mm.   Basal piece = 30 mm.
Series B   Apical piece = 30 mm.   Basal piece = 10 mm.

SERIES A			SERIES B		
Number of individual	Apical piece 10 mm.	Basal piece 30 mm.	Number of individual	Apical piece 30 mm.	Basal piece 10 mm.
1	99	73	1	79	79
2	69	69	2	67	71
3	69	67	3	71	73
4	67	67	4	77	77
5	69	69	5	71	69
6	67	67	6	71	69
7	67	67	7	79	91
8	67	67	8	91	91
9	69	69	9	91	91
10	73	71	10	67	77
11	69	69	11	69	73
12	67	67	12	77	77
13	91	71	13	67	71
14	71	71	14	67	71
15	77	71	15	77	77
16	69	69	16	69	71
17	77	67	17	73	73
18	67	67	18	71	91
19	67	67	19	69	71
20	69	71	20	77	79
Averages.......	72.0	68.8		73.9	77.1
Differences ....3.2 hours					3.2 hours

We took two series of twenty stems each; in series A the shorter piece of 10 mm. was the apical one and the longer of 20 mm. was the basal one; while in series B, on the contrary, the shorter piece was the basal one and the longer the apical one.   The averages are in series A: for the apical piece (10 mm.) 65.4 hours, and for the basal piece (20 mm.) 64.2 hours; in series B: for the apical piece (20 mm.) 69.0, and for the basal piece (10 mm.) 70.3 hours.   The oral hydranth of the larger piece emerges about an hour earlier than that of the smaller pieces, a difference that

TABLE 5

Time in hours required for the emergence of the oral hydranths in pieces of different length

Ratio of the length of the pieces = 1 : 4.
Series A   Apical piece = 10 mm.   Basal piece = 40 mm.
Series B   Apical piece = 40 mm.   Basal piece = 10 mm.

SERIES A			SERIES B		
Number of individual	Apical piece 10 mm.	Basal piece 40 mm.	Number of iudividual	Apical piece 40 mm.	Basal piece 10 mm.
1	72	72	1	70	94
2	70	70	2	76	82
3	94	74	3	74	82
4	72	72	4	74	70
5	74	70	5	82	78
6	94	94	6	94	74
7	74	78	7	74	82
8	98	74	8	94	74
9	118	70	9	72	74
10	70	70	10	—	—
11	82	96	11	72	82
12	64	54	12	—	—
13	98	94	13	70	70
14	82	106	14	70	74
15	70	82	15	72	82
16	94	96	16	70	94
17	—	74	17	70	78
18	102	72	18	76	—
19	94	94	19	72	82
20	70	70	20	70	94
Averages.......	83.8	79.1		75.1	80.0
Differences................4.7 hours					4.9 hours

is very small indeed but the same in both series.   There was no influence of the level factor.

In the experiment recorded in table 4, the ratio of the lengths of the pieces was 1 : 3.   In series A the shorter piece of 10 mm. is the apical one and the longer piece of 30 mm. is the basal one, while in series B the reverse occurs.   The averages are in series A: for the short apical piece 72.0 hours, and for the longer basal piece 68.8 hours; in series B: for the longer apical piece 73.9 and for the shorter basal one 77.1.   The differences between the

time of regeneration of the shorter and the longer pieces are 3.2 hours in favor of the larger piece in both series.

In table 5 the ratio of the sizes of the pieces was 1 : 4 and the averages are in series A: for the shorter apical pieces 83.8 hours, and for the longer basal one 79.1 hours, with a difference of 4.7, while in series B the averages are: for the longer apical piece 75.1 hours, and for the shorter basal piece 80.0, with a difference of 4.9.

In any of these cases there was no evidence of the existence of level or regional differences on the stems of Tubularia.

CONCLUSIONS

Child has based his theory of the 'metabolic gradients' on the assertion that if a stem of Tubularia is cut into two pieces the oral end of the apical piece will regenerate a hydranth more rapidly than the oral end of the basal piece. The differences in the time of regeneration observed by him were so small that they seemed to lie within the limits of error of such experiments.

The writer repeated Child's experiments and found this suspicion justified. The rate of regeneration of the oral hydranth of an apical piece is on the average identical with the rate of regeneration of the oral hydranth of the basal piece. If in one series the average is in favor of the apical piece, in another the reverse may be found.

There is no evidence of the existence of level or regional differences of the rate of regeneration in the stem of Tubularia, and as a consequence there is no basis for the theory of 'axial gradient' in this species.
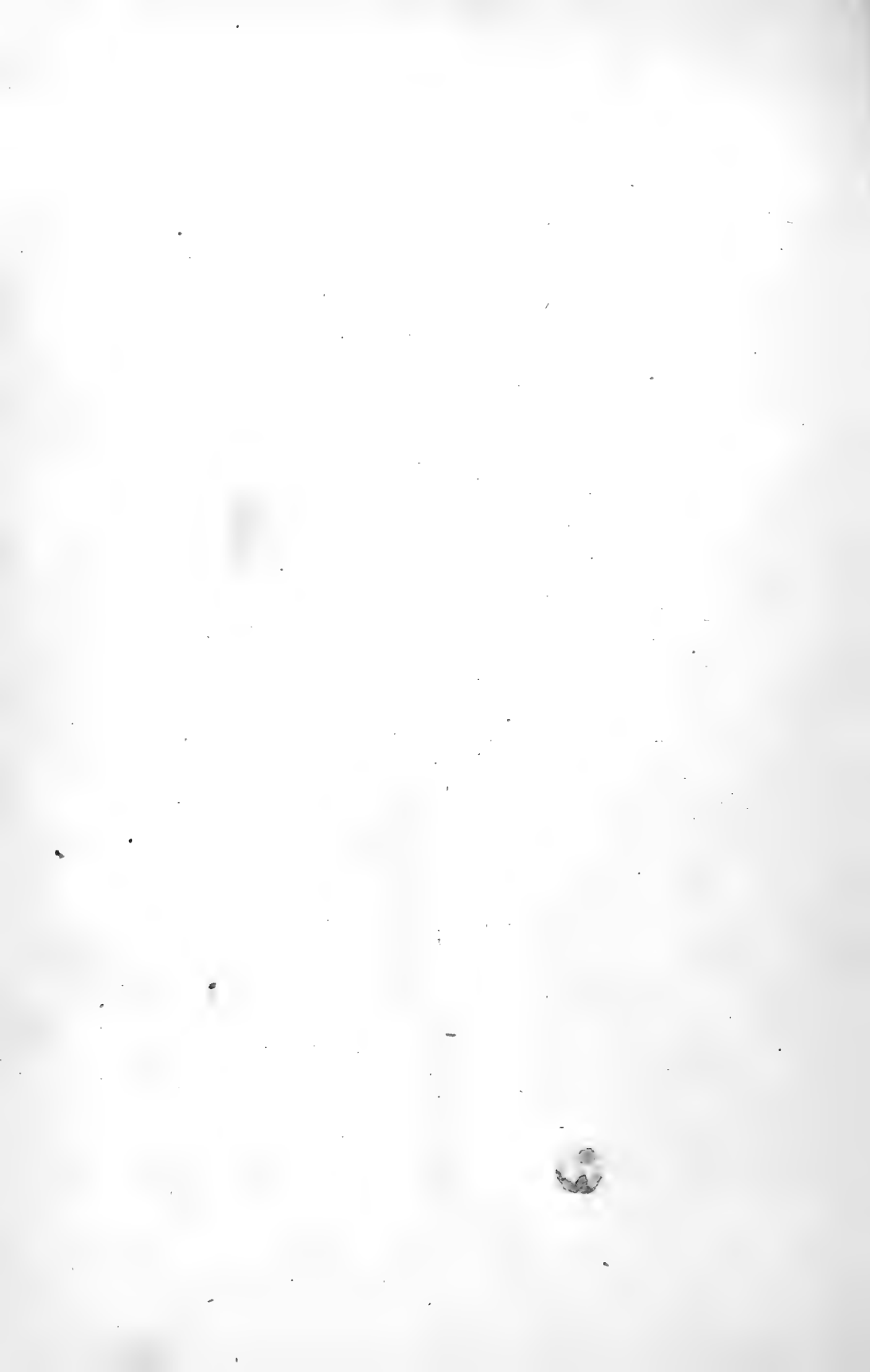
THE PHYSIOLOGY OF THE MELANOPHORES OF THE HORNED TOAD PHRYNOSOMA[1]

ALFRED C. REDFIELD

From the Zoölogical Laboratory of the Museum of Comparative Zoölogy at Harvard College

EIGHT TEXT FIGURES AND FIVE PLATES

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I. INTRODUCTION

The results of an investigation of the reactions, coördination, and function of the melanophores of the horned toad Phyrnosoma cornutum Harlan are described in the following paper. The facts which are presented possess, in addition to their intrinsic interest, a bearing upon several aspects of comparative physiology.

Earlier studies upon the melanophores of lizards indicated that in the chameleon (Brücke, '52; Keller, '95) the color changes are produced under the influence of nerves which cause the melanophore pigment to contract, while in Anolis (Carlton, '03) the nerve impulse appears to cause the opposite effect, an expansion of the melanophore pigment. The present investigation was initiated in the hope of gaining more light upon the relation of the nervous system to the melanophores. It very soon became evident that the melanophores are not only under the control of nerves, but that certain of their reactions result from the direct effect of stimuli upon them, while other reactions indicate clearly that some coördinating mechanism other than the nervous system is involved. The discovery that these reactions are due to a hormone (Redfield, '16) and the identification of the hormone as adrenin form the most novel result of the investigation.

A few observations have already been made upon the color changes of this lizard by de Grijs ('99) and upon the closely related species Phrynosoma orbiculare, by Wiedersheim (Hoffmann, '90, p. 1353), and Phrynosoma modestum, by Weese ('17). Parker ('06) has made a more intensive study of the reactions of the melanophores of Phrynosoma blainvillei to illumination

and temperature.   The great mass of data upon the chromatophores of other lizards has been reviewed recently by von Rynberck ('06) and by Fuchs ('14).

Horned toads have been obtained from collectors in various parts of Texas and Oklahoma.   They may be had in large numbers between April and September.   In the laboratory they were kept in a large sunlit cage, where they thrived on a diet of meal worms.   In summer the animals were kept in cages out of doors and fed on various insects in season.   Horned toads do not feed if kept in the laboratory through the winter.   They become greatly emaciated and usually die before spring   If these lizards are placed in a dark, cool cellar, they go into hibernation and remain in very good condition all winter.

Experiments have been carried on in the Zoölogical laboratory of Harvard College at Cambridge, Massachusetts, in the physiological laboratory of the Harvard Medical School in Boston, and in the laboratory of the United States Bureau of Fisheries at Woods Hole.   I wish to acknowledge my indebtedness to Dr. G. H. Parker for his kindness in directing this investigation and to Dr. Walter B. Cannon for valuable advice on certain phases of the work.

II. THE MELANOPHORE REACTIONS OF THE HORNED TOAD

1. Description of color changes

Under certain conditions the ground color of the upper surface of the horned toad is fuscous.[2]   Across the back run three irregular bands of fuscous-black, bordered posteriorly with a bright amber-yellow line, which stands out prominently in contrast to the dark ground-color.   Similar fuscous-black bands, not bordered with yellow, extend across the legs.   The flattened scales which extend in a row along the sides of the body are black at the base, white at the tip.   Under other conditions, the ground-color becomes drab, drab-gray, or cinnamon-buff, frequently flecked with black.   The amber-yellow lines across the back no

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[2] The color nomenclature of Ridgway ('12) is used throughout this description.

longer stand out in contrast with the ground-color, but the fus-
cous-black bands now become very prominent both on the back
and legs.   The lateral scales become white throughout, with the
exception of two on each side which are located at the ends of the
fuscous-black cross bands of the back.   These scales rarely be-
come entirely free from pigment.   Plate 1 illustrates the extremes
of these color changes.[3]

The color changes of lizards have been shown by Brücke ('52),
Keller ('95), and Carlton ('03) to be due to the migration of pig-
ment contained in cells known as chromatophores, which are
situated in the dermal layer of the skin.   An examination of
sections of the skin of the horned toad reveals the presence of
chromatophores, filled with black pigment, such as Keller ('95)
has called melanophores.   When the melanophore pigment
expands the ground-color becomes fuscous; when it contracts
this portion of the skin becomes drab.   The fuscous-black bars
across the back and legs contain such an abundance of non-
motile pigment that their color does not change.

In addition to the melanophores there exist in the skin pigment
cells containing a yellow pigment.   Whether this pigment is
motile has not been determined, certainly it causes no prominent
color changes.

2. Daily rhythm of color changes

At night the melanophore pigment of the horned toad is con-
tracted, giving the lizards a pale appearance.   In the early
morning this pigment expands and the skin becomes uniformly
dark.   During the heat of midday the melanophore pigment
contracts again, but as evening approaches there is a second ex-
pansion, followed finally by a contraction once more as night
sets in.   Thus the pigment is contracted and the animals pale
at night and at midday; the pigment is expanded and the animals
dark in the morning and afternoon.

[3] Photographs fail to bring out fully the contrast between the dark and the pale
condition of the skin.   This is because the skin is tinged with yellow, a non-actinic
color.   Orthochromatic plates and a color screen have been used, but without
complete success, in an attempt to overcome this difficulty.

3. Adaptive color changes

Horned toads show striking color changes which depend upon the color of the environment in which they live. If they are placed in a pen which is lined with black cinders, the ground-color becomes so dark that the transverse bands are almost indistinguishable. When such animals are transferred to a pen lined with white sand, the ground-color becomes very pale in the course of a few days. The contrast between these pale animals and horned toads which have been left in the cinder-lined cage as controls is illustrated in plate 1. This change in color becomes noticeable within one day after the lizard has been placed in its new environment, and reaches a maximum within one week.

Upon the adaptive state of the chromatophores is superimposed the daily rhythm of color change. Thus horned toads which are adapted to a dark background become paler at night and at midday, but never attain the extreme paleness of specimens adapted to a light background. Similarly, horned toads which are adapted to a light background become darker in the morning and afternoon, especially on cool days, but never attain the dark color of specimens which are adapted to a dark environment.

4. Color changes due to nervous excitement

Whenever horned toads are thrown into a state of nervous excitement the melanophore pigment throughout the entire surface of the body is contracted and the animal assumes the maximal pale condition. This reaction is observed whenever one of these lizards is subjected to any treatment which causes it to struggle in an effort to free itself. Simply holding a horned toad upon its back, attempting to pry open its mouth, or pinching its tail may suffice to cause this reaction. A male horned toad, attempting to copulate with a female, has been observed to become very pale at a time when the pigment of the other animals in the pen was expanded fully.

The contraction of the pigment which accompanies nervous excitement occurs more rapidly than the two other types of reaction which have been described. The complete change in color

may occur in as short a time as three minutes and never requires longer than ten minutes. This reaction is also a dominant one, occurring irrespective of the condition which the cycle of day and night and the color of the environment has imposed upon the melanophores.

III. EXTERNAL STIMULI INVOLVED IN MELANOPHORE REACTIONS

1. *Illumination and temperature*

The series of color changes which accompanies the cycle of day and night is dependent upon the accompanying changes in light and temperature. The rhythm of the color changes may be broken by a disturbance of the normal rhythm of the changes in light and temperature. Thus on cool days the midday contraction of the melanophore pigment does not occur; on cloudy days or when the animals are buried in the sand the skin may remain in the pale condition throughout the entire day.

If a horned toad is exposed to bright daylight or to sunlight which is not too warm, after a short time an expansion of the melanophore pigment occurs. If the animal is placed in the dark, the melanophore pigment is contracted. These changes may be followed most clearly by observing the appearance and disappearance of the dark areas upon the bases of the lateral scales. The rapidity with which these changes occur in either direction varies from ten minutes to one-half hour, depending upon the intensity of the illumination, the temperature, and the idiosyncrasy of the individual.

In order to determine whether the effect of light upon the melanophores is due to the heat developed by its action, the following experiment was performed. An apparatus was devised in which the temperature could be kept quite constant and to which light could be admitted at will. This apparatus consisted of a glass aquarium 30 cm. in diameter and of equal height. Into this was inserted a second glass vessel 15 cm. in diameter. Between the walls of these two jars ice or water of any desired temperature could be introduced. By this means the air in the

inner jar could be kept within a degree or two of a given temperature.   In the inner jar was placed a layer of sand, which served to keep it floating upright and gave a natural support for the animal.   This jar was kept covered to maintain the temperature and humidity as constant as possible.   When desired light was excluded from this apparatus by inverting over it a large pasteboard box.

November 26, 1913.   Two horned toads, the melanophore pigment of which was expanded, were placed in the apparatus at 10.45 A.M. They were illuminated by the light from the overcast sky.   Warm water was placed in the outer jar.
    After one hour (11.45 A.M.) the temperature of the air in the inner jar had risen to 40°C. and the melanophore pigment of the animals had *contracted*.
    One-half hour later (12.15 P.M.), the temperature of the air in the inner jar had fallen to 26°C. and the melanophore pigment had *expanded slightly*.   Ice was added to the water.
    Nearly two hours later (2.00 P.M.), the temperature was found to be 16°C. and the pigment was *fully expanded*.   The apparatus was then covered with a box to exclude the light.
    After one-half hour (2.30 P.M.) the melanophore pigment was still *expanded*.   Temperature 17°C.   The ice-water was replaced with warmer water.
    One hour later (3.40 P.M.) the temperature had risen to 25°C. and the pigment had *contracted* again.   It remained in this condition as the temperature was raised to 36°C. at 4.20 P.M.

Repeated experiments confirm the conclusions which may be drawn from these data.   It makes no difference in which order the changes of temperature and illumination are arranged.

The following summary indicates the condition of the melanophore pigment under various conditions of temperature and illumination:

1. High temperatures produce a contraction of the melanophore pigment, irrespective of illumination.

2. Low temperatures produce an expansion of the melanophore pigment, irrespective of illumination.

3. At intermediate temperatures (30° to 20°C.) the state of the melanophores is conditioned by the illumination, light causing an expansion and darkness a contraction of the pigment.

Light and heat thus act in opposite ways and their effects need not be confused.   The expansion of the melanophore pigment in the light cannot be due to heat, as this stimulus causes a contraction of the pigment.

In certain individuals light dominates over temperature in determining the state of the melanophores through a larger range of temperatures than that indicated above.   Some horned toads maintain a contracted condition of the pigment in the dark at temperatures as low as 5°C.   The pigment of others may expand upon illumination when confined at temperatures as high as 37°C.

These conclusions are in very good agreement with those which Parker ('06) drew from experiments upon Phrynosoma blainvillei. By their means may be explained the rhythmic changes in the color of horned toads which are correlated with the cycle of day and night.   The expansion of the pigment in the morning is due to the stimulation of light.   The heat of midday causes a contraction in spite of the light, but as the air cools in the afternoon the light effect again dominates and the pigment expands.   When the light fails, at night, the pigment becomes contracted as a result.

2. The color of the substratum

It has been pointed out on a preceding page that the color of the environment has a marked effect upon the condition of the melanophores.   A dark substratum produces an expansion of the pigment in horned toads which live upon it; a light colored substratum has the reverse effect.   The color of the substratum must consequently be considered the stimulus which initiates the adaptive changes in the color of this animal.

This stimulus must not be confused with the effect of illumination upon the melanophore.   The reaction which it produces is quite a different phenomenon, as the following considerations indicate.   The results of the two forms of stimuli are diametrically opposed; light causes an expansion, a light background a contraction of the pigment.   The photo-receptors concerned with the two sources of stimulation are quite distinct, as will be shown in another place.

3. Mechanical stimuli

Mechanical stimulation, such as tapping or pressing the skin, does not produce any effect upon the melanophores. Mechanical stimuli of a more violent nature cause profound changes in the condition of the melanophores. Such stimuli, however, do not act directly upon the pigment cells to which they are applied, but affect the pigment of the entire body by producing a state of nervous excitement. Such stimuli are better classed with noxious stimuli, to be considered later.

4. Faradic stimuli

The application of a faradic stimulus to the surface of the skin of the horned toad fails to produce a change in the state of the melanophores. Apparently the dry horny layer of the skin forms a very poor conductor for the electric current. If the electrodes are applied to the under surface of the skin, the melanophore pigment of the region becomes completely contracted in the course of five minutes. Faradic stimuli, if sufficiently violent, may produce effects upon the melanophores of the entire body. Such stimuli are conveniently classed as noxious stimuli.

5. Noxious stimuli

In this category may be placed a variety of stimuli which have a common effect upon the state of the nervous system of the horned toad. Noxious stimuli are those which may be considered harmful, painful, or otherwise unpleasant and produce in the horned toad a state of nervous excitement. Whenever such a nervous condition is established in this animal there ensues a complete contraction of the melanophore pigment. A description of some of the conditions which produce nervous excitement has already been given on page 279. For experimental purposes the most convenient noxious stimulus is the electric current. By placing platinum-point electrodes in contact with the mucous surface of the mouth or cloaca of the animal and passing a faradic current between these points, a complete contraction of the pig-

ment may be produced. The current used for this purpose was just strong enough to cause an unpleasant sensation when applied to the human tongue.

Summary

The responses of the melanophores of the horned toad to external stimuli are the following:

1. Light produces an expansion; its absence a contraction of the melanophore pigment.

2. High temperatures produce a contraction; low temperatures an expansion of the melanophore pigment.

3. Light and heat interact in such a way that the heat effect dominates at extremes of temperature, the light effect dominates at mean temperatures.

4. Light coming from a dark substratum produces an expansion of the pigment; light coming from a light substratum produces a contraction of the pigment.

5. Mild mechanical stimuli do not affect the melanophores.

6. Mild faradic stimuli cause a contraction of the melanophore pigment.

7. Noxious stimuli, such as violent mechanical or faradic stimuli, produce a contraction of the melanophore pigment.

IV. RECEPTORS INVOLVED IN MELANOPHORE REACTIONS

1. Direct action of stimuli upon melanophores

It has been pointed out by Spaeth ('13) that the direct action of stimuli cannot be studied satisfactorily by experiments upon living animals, owing to the complications introduced by the presence of the nervous system, circulation, etc. He recommends the study of bits of tissue separated from the body of the animal. Unfortunately, the horned toad does not lend itself easily to this method of investigation. Pieces of skin placed in Ringer's solution show no melanophore reactions, except that the pigment is contracted very soon. This contraction may be attributed to anemia (p. 292).

By means of carefully controlled experiments, however, it is possible to gain some idea of the significance of the direct action of stimuli upon the melanophores without removing them from the organism. If these pigment cells react in only those places on which stimuli act, a direct response of the melanophore may be suspected; if the pigment throughout the entire body is affected by a local stimulus, it may be concluded that the reaction is due to the intermediation of nerves, or hormones. The following experiments are instructive from this point of view:

November 1, 1913. Three horned toads, the pigment of which was contracted, were shielded locally from light by placing a small piece of modeling clay over two or three lateral scales. After an hour of exposure to sunlight the melanophore pigment of all the lateral scales of all the animals had expanded, with the exception of the shielded scales, the pigment of which remained contracted.

Two horned toads, the melanophore pigment of which was expanded, were shielded in a similar fashion and exposed to sunlight. The melanophore pigment of all the scales remained expanded *excepting those shaded by the clay, which contracted.*

From this experiment, which has been amply confirmed, it is evident that if a small part of the surface of a horned toad be shielded from light, the melanophore pigment of this part will remain contracted while that of the rest of the body expands, and if the shaded melanophore pigment be expanded, it will contract without reference to the illumination of the rest of the body.

It might be objected that this effect is due to the tactile stimulation of the clay or to the exclusion of air from the surface of the skin. That this objection is invalid is indicated by the fact that if the clay is applied so as not to touch the lateral scales, but only the surface of the skin on both sides of it, the reaction follows in the same manner. Moreover, if the scales are painted with celloidin, which should provide a similar tactile stimulation and exclude the air from the skin, but which does not keep out the light, the pigment is able to expand as readily as on those scales which are not covered.

The converse experiment consists in illuminating a few scales of the body when the remaining parts are covered. This experi-

ment has been performed in various ways and has yielded variable results. In a few cases the outcome has been in good agreement with the expectation raised by the experiment just described.

August 24, 1915. A horned toad, having the melanophore pigment expanded, was tied to a board and covered with a piece of black woolen cloth. Through a hole in this cloth a portion of the right half of the back was exposed to the light.

2.45 P.M. Placed in sunlight.

3.00 P.M. Exposed part of back unchanged. Melanophore pigment of unexposed part of skin is contracted.

3.15 P.M. Body completely covered with black woolen cloth.

3.30 P.M. Melanophore pigment of entire skin contracted.

August 24, 1915. A horned toad, having its melanophore pigment completely contracted, was tied to a board and covered with black woolen cloth. Through a hole in the cloth 1 sq. cm. of the anterior part of the left side of back was exposed to the light.

2.40 P.M. Placed in sunlight.

3.00 P.M. Melanophore pigment of exposed part of skin has expanded; unexposed skin is still pale.

From these experiments it is again evident that the illumination of a restricted area of the skin will cause an expansion of the pigment of that area, without regard to the illumination of the rest of the body or to the reactions of melanophores of the other parts of the skin.

Similar experiments have been performed in which heat was used instead of light as a local stimulus. The temperature of a few lateral scales could be modified by the following method. Against the side of the body of a horned toad a nozzle was applied through which ran a current of water of the desired temperature. The nozzle was made of a piece of glass tube 2 cm. in diameter. In one end was inserted a rubber stopper carrying an inlet and outlet tube and a thermometer. Over the other end a piece of sheet rubber was tied loosely. When pressed against the skin of a horned toad, the rubber end of the nozzle wrapped itself snugly about the scales and soon imparted to them a temperature which must have approximated that of the water within the nozzle.

December 15, 1913. Water at a temperature of approximately 45°C. was passed through the nozzle applied to the right side of a horned toad the melanophore pigment of which was expanded. The experiment was performed in the dark so that the exclusion of light from the skin by the

nozzle could not form a disturbing factor. After three minutes the right side was much paler than the left; after six minutes the melanophore pigment of the right side was completely contracted. The left side remained unchanged until the nozzle was applied to it, when it, too, became pale.

This experiment, repeated on a number of animals, indicated that if a portion of the skin of a horned toad is heated while in the dark, the expanded melanophore pigment of that portion will contract much more rapidly than that of the other parts of the body. That this result is not produced by the contact of the apparatus, but is a true temperature effect, is shown by the fact that the application of the nozzle to the skin of a horned toad does not produce a more rapid contraction of the melanophore pigment of that part of the skin if the water within the nozzle is at room temperature.

The converse experiment, the application of a low temperature to a small part of the skin, is illustrated in the following protocols:

December 18, 1913. A nozzle through which ice water was running was applied to the side of a horned toad the melanophore pigment of which was contracted. Throughout thirty minutes of this treatment, during which the animal was kept in the dark, no change occurred in the condition of the pigment cells.

In the same way a low temperature (10.5°C.) was applied to the side of a horned toad of which the melanophore pigment was expanded. The animal was in the dark. After six minutes the melanophore pigment of the animal had contracted, as might be expected in the dark. The melanophore pigment of the chilled portion of the skin *did not contract* in twice that time.

From these experiments it appears that the local application of low temperatures to the skin of the horned toad cannot call forth an expansion of the melanophore pigment,[4] but that an expansion previously established may be maintained in the region of low temperature, though the rest of the skin becomes pale.

[4] The action of a low temperature when applied locally to the melanophores is discordant with the results of applying light and heat and excluding light locally. It may be pointed out that the facts are no more readily explained by the assumption that nerves are concerned in the reaction, so that the point does not argue for or against the ability of the melanophores to respond directly to the stimulation of light and temperature.

To summarize the effect of local photic and thermal stimuli upon the melanophores of the horned toad, the following points should be brought out:

1. The local exclusion of light and the local application of heat, both of which produce a contraction of the melanophore pigment in the parts so treated, do not affect the melanophores in other parts of the body.

2. The local application of light, which produces an expansion of the exposed melanophore pigment, does not affect the melanophores of other parts of the body.

3. The local application of a low temperature fails to produce any expansion of melanophore pigment. An expansion previously established may be maintained locally by this stimulus.
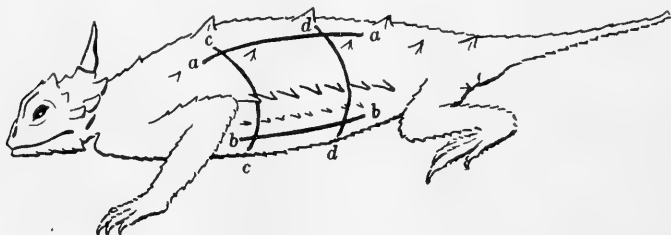
Fig. A   For explanation see text (p. 288).

These experiments do not prove that the melanophores are stimulated directly by light and heat, because the same results might be obtained by a different mechanism. If the melanophores were under the control of nerves so arranged that the stimulation of a point on the skin sets up impulses which passed over a reflex arc terminating in the pigment cells of the same point and no other, then a local stimulus would also produce a local response.

An experiment designed to test the presence of such a system of reflex arcs consisted in making cuts through the body wall of a number of horned toads in such positions that had all the cuts been made upon a single animal a part of the skin would have been completely severed from the rest of the body. Thus one animal received a cut in a position indicated by the line a–a, in figure A ; a second, third and fourth animal in positions indicated

by the lines *b–b*, *c–c*, and *d–d* respectively.   Whatever the course
of any nerves connected with the melanophores within the region
inclosed by these four lines, one of the cuts must be expected to
interfere with the passage of nervous impulses to and from the
region.   The animals upon which these operations were performed
recovered rapidly.   Four days after the operation, when the
melanophore pigment was fully expanded, they were placed in the
dark.   Within two hours the melanophore pigment of all parts
of the skin in every one of the four cases had contracted.   Since
this contraction cannot be attributed to a nervous reflex, the con-
clusion must be drawn that light acts directly upon the melano-
phores of the horned toad.[5]   In a similar way it was found that a
high temperature would cause a contraction of the melanophore
pigment of all parts of the skin of these animals.

It is clear that a local response to local stimuli cannot be
explained by postulating the action of hormones or of a diffuse
nervous mechanism, such as a nerve net.   It has been shown that
these responses cannot be attributed to a nicely arranged system
of reflex arcs.   The conclusion seems unavoidable, therefore, that
the action of light and temperature upon the melanophores of the
horned toad is a local one, which does not involve the coördinat-
ing systems of the body.

2. Receptors of noxious stimuli

Noxious stimuli can bring about melanophore reactions when-
ever they affect sensory nerves with sufficient intensity.   It has

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[5] The exact point upon which these stimuli act is still a subject for speculation.
The simplest conception is that light and cold stimulate the melanophores directly
and so produce their characteristic effects.   If this be true, the melanophores
may then be considered to serve as receptors for these stimuli.   This conception,
however, may be naive.   Light and heat may act not directly upon the melano-
phores, but upon some closely associated tissue and still bring about the observed
results.   Thus it will be shown (page 306) that the melanophores are acted upon
directly by nervous impulses, which maintain a contraction of their pigment.
Assume that cold acts upon the motor nerve endings which connect with the
melanophores so as to block the passage of nerve impulses to the pigment cells,
and the expansion of the melanophores by cold may be explained as an inhibition
of the nervous control, rather than a direct positive effect of the stimulus upon the
pigment cells.   The present investigation has offered no grounds for choice
between the two possible mechanisms.

been found that an induced current of electricity will stimulate the terminations of sensory nerves which are found in the month and cloaca and thus bring about a contraction of the melano-phores. Stimulation of receptors in the ear are probably responsible for setting up the state of nervous excitation which produces a contraction of the melanophores when the horned toad is held upon its back. One cannot say exactly what nerves are stimulated when an attempt is made to pry open the mouth of a horned toad, but this procedure also sets up a nervous disturbance which produces a contraction of the melanophore pigment. It seems clear that there are no specific receptors involved in the reaction; any receptor, the stimulation of which produces the necessary state of nervous excitement, is capable of initiating the reaction by which the melanophore pigment is contracted.

3. Receptors involved in adaptive reactions

The reactions of the melanophores to the color of the substratum upon which the horned toads are kept depend upon stimuli received through the eyes. If horned toads which have been kept upon a bed of dark cinders are transferred to a bed of white sand, they become noticeably paler after one day and reach the maximum state of contraction of the melanophore pigment within five days. If such animals are blindfolded by fastening bits of woolen cloth over their eyes with celloidin and adhesive tape, they will retain the dark color, although they are kept upon a light colored background for several weeks.

The failure of the melanophore pigment to contract is not due to any influence of the bandage, for animals which have had only one eye bandaged become pale as rapidly as specimens which have not been bandaged at all. Plate 2 shows a number of animals which illustrate such an experiment. In figure 3, a is a horned toad which has been kept upon cinders, b an animal which has been kept upon sand without blindfolding. The contrast in the pigmentation occurring under the two conditions is obvious. Figures 4 and 5 show four horned toads all of which have been upon sand for ten days: d and f, having had the use of their eyes,

have become pale; c and e, having been blindfolded, remain as dark as the animals which have been kept upon cinders. Figure 6 shows a control experiment, in which both animals have been upon white sand for ten days; g was blindfolded in one eye, yet it has become as pale as h, which was not blindfolded.

This experiment leaves no doubt that the eyes are the receptors involved in the adaptive reaction of the melanophores to the color of the environment.

Summary

1. The melanophores or some closely associated tissues are receptors of photic and thermal stimuli.

2. There are no specific receptors for noxious stimuli.

3. The eyes are receptors for stimuli which cause adaptive reactions of the melanophores.

V. COÖRDINATING MECHANISMS OF MELANOPHORE REACTIONS

In the foregoing pages there have been considered the stimuli which initiate reactions of the melanophores of the horned toad and the receptors upon which these stimuli act. There remain to be considered the mechanisms involved in transmitting the effect of a stimulus to the pigment cells. By what means are the reactions of the melanophores coördinated, so that a localized stimulus can cause a change in the color of the entire skin? Obviously this question need not be answered for those reactions in which the melanophores or some closely associated tissues are stimulated directly, as by light and heat. Noxious stimuli and stimuli arising from the color of the environment, however, may act on a restricted sensory area and yet cause a reaction of pigment cells over a large surface of the body. How is this integration accomplished?

Two systems of the body are concerned in coördinating its parts. These are the circulation and the nervous system. The relation of these systems to the reaction of the melanophores of the horned toad will now be considered.

1. Coördinative action of the circulation

a. Influence of the respiratory function of the circulation upon melanophores. The circulation of the blood is obviously important in maintaining the metabolic activity of the melanophores. Stoppage of the circulation might be expected to produce effects upon the state of the melanophores, either on account of failure in the oxygen supply which must result or because of the accumulation of carbon dioxide or other katabolic products in the tissues. This expectation is readily realized.

If a ligature is tied tightly about the leg of a horned toad, the melanophore pigment of which is expanded, no immediate result follows. In the course of two hours the skin of the ligatured leg becomes very much paler than that of the rest of the body (fig. 7). Evidently the stoppage of circulation has caused a contraction of the pigment. Upon removing the ligature the circulation is restored, and after an hour and a half the melanophore pigment becomes expanded once more so that the color of the leg is indistinguishable from the other parts of the skin.

If small bits of skin containing expanded melanophore pigment are cut from a horned toad and placed in a normal salt solution, this pigment becomes contracted very rapidly, usually within five or ten minutes. The skin of the horned toad also becomes pale within a short time after the death of the animal.

The contraction of the melanophore pigment in these cases seems to be due to the interruption of the circulation. Whether the reaction is due to a deficiency of oxygen or to an accumulation of carbon dioxide or other waste materials in the tissue has not been determined. The effect of anemia upon the melanophores probably is not a thing which comes into play in producing any of the usual melanophore reactions. Consequently, the other mode of action of the circulation upon these cells will prove of greater interest.

b. The coördination of melanophores by hormones. The circulation of the blood is known to influence the tissues to which it flows by carrying to them chemical substances, such as hormones, which excite the tissues to various forms of activity. In

attempting to discover the mechanism through which a noxious stimulus, locally applied, is able to produce a general contraction of pigment of the entire body, it became apparent that the melanophores are coördinated by means of a hormone (Redfield, '16).

When the nerves supplying a region of the skin are cut, and the surface of the mouth or cloaca is stimulated with a weak faradic current, the melanophore pigment of the entire surface of the skin contracts. There is no difference between the behavior of the melanophores in the part which has been denervated and the rest of the skin. For example, the sciatic nerve may be cut close to the junction of one hind leg with the trunk. Upon applying faradic stimulation to the mouth, both hind legs become equally pale as the melanophore pigment of the entire skin contracts. Again, the series of horned toads operated upon in the manner described on page 288 may be subjected to a noxious stimulation. In none of these animals does the operation, which in some cases at least must have cut the nerves to the portion of skin under observation, interfere with the contraction of the pigment cells upon this region of the body. It is clear that the reaction may be completed without the aid of nerves which connect directly with the melanophores. A hormone, set free through the action of the noxious stimulus, is the natural alternative to account for this result.

To test the hypothesis that the pigment is contracted by a hormone, carried to it by the circulation, the following experiment was tried:

August 30, 1915. Tied a ligature firmly about the right hind leg of a very dark horned toad. Movements of the lower part of this leg indicated that pressure of the ligature had not blocked the nervous impulses to the leg.

12.20 Started stimulating mouth with weak faradic current.

12.25 Color of skin of right leg unchanged. Skin of left leg has become much paler, in striking contrast to the right leg (fig. 8). Melanophore pigment of the other parts of the body has contracted. Stopped stimulation.

12.27 Removed ligature.

12.36. Right leg has become as pale as the left.

From this experiment, which has been repeatedly confirmed, it is apparent that by blocking the circulation to one leg of the horned toad without interfering with the innervation of the leg noxious stimuli are prevented from influencing the melanophores of that leg. This result is to be expected if the melanophore pigment is contracted by a hormone liberated in the circulation by noxious stimuli.

The fact that the melanophore pigment of the isolated leg becomes contracted upon the removal of the ligature (fig. 9), several minutes after the cessation of stimulation, confirms the hypothesis further.

If noxious stimuli cause the production of a hormone in the blood which contracts the melanophore pigment, one might expect that blood drawn from a horned toad which had been thrown into a state of nervous excitement by this means would cause the contraction of the pigment of an unexcited animal when injected into its body. How far this expectation is realized is indicated by the following account of such an experiment:

July 28, 1915. 2.00 P.M. Needle of hypodermic syringe inserted into the lymph space under the skin of the right side of a horned toad the melanophore pigment of which was expanded fully.

2.52 P.M. Through this needle was injected 0.5 cc. of defibrinated blood drawn from the neck of a second horned toad, which had been made very pale by stimulating the mouth with a weak faradic current for ten minutes.

3.04 P.M. The skin of the first horned toad has become *clearly pale along the right side of the back* (fig. 10). The melanophore pigment of some lateral scales near the point of insertion of the needle has contracted.

3.18 P.M. No change.

3.28 P.M. Pale patch is becoming darker.

3.50 P.M. Pale patch is almost indistinguishable from the rest of skin.

7.00 P.M. Pale patch has entirely disappeared.

This experiment indicates that the blood of an excited horned toad will cause a contraction of the melanophore pigment of an unexcited animal. The size and intensity of the pale area varied considerably in different experiments. In no case did it extend beyond the side of the body into which the blood was injected.

The foregoing experiment must be carefully controlled, for it is quite conceivable that blood from any horned toad might have an effect upon the melanophores of another individual, irrespective of the presence of hormones in it. That this supposition is untenable is indicated by the following experiment:

August 4, 1915. 12.30 P.M. Hypodermic needle inserted into the lymph space under the skin of the left side of a horned toad the pigment of which is expanded.
1.42 P.M. Injected through the needle 0.2 cc. of defibrinated blood drawn by quickly decapitating a second horned toad. This decapitated animal had had a portion of the thoracic spinal cord destroyed, an operation which prevents any noxious stimulation from producing a contraction of the melanophore pigment (see p. 300). In this way the presence of the suspected hormone is avoided. The first animal still had its pigment expanded.
1.53 to 3.00 P.M. No change in the color of the first horned toad.

Since the exclusion of blood from a part of a horned toad during excitement causes the melanophore pigment of that part to remain expanded, and since the injection of blood from an excited horned toad into an unexcited individual causes the melanophore pigment of the latter to contract, it may . be concluded that the melanophores of the horned toad are influenced by a hormone, present in the blood during nervous excitement, which causes the melanophore pigment to contract.

c. The nature of the hormone involved in melanophore reactions. What is the chemical substance which causes a contraction of the melanophore pigment of the horned toad and where is it produced?

The conception of a hormone regulating the activity of melanophores is not altogether novel. Fuchs ('14, pp. 1546 to 1547, 1651 to 1652) has attempted to explain the behavior of pigment cells in amphibian larvae and reptiles by assuming that substances, perhaps internal secretions, which contract the pigment, are produced in the body under the regulation of the pineal organ. Laurens ('16) has recently shown this hypothesis to be inapplicable to the phenomena observed by him in Ambylstoma punctatum.

The pineal body of the horned toad (Phrynosoma douglassii and Phrynosoma coronata) has been described by Ritter ('91),

who finds it to be a highly developed organ, in part closely
resembling an eye. The structure of the epiphysis, with its
folded epithelial walls containing great quantities of blood cor-
puscles, does not forbid the interpretation that it may function
as a gland of internal secretion. It may be readily shown, how-
ever, that it is not the organ concerned, primarily at least, with
the production of the melanophore hormone. The skull of a
horned toad may be trephined and the entire brain anterior to
the cerebellum, and including the pineal organ, may be removed.
The animals recover from this operation and their pigment be-
comes fully expanded. Stimulation of the cloaca with a weak
induced current is still able to produce a contraction of the
melanophores.

A more favorable clue to the identity of the melanophore hor-
mone is found in the work of Cannon and his collaborators
(Cannon, '15). Cannon and de la Paz ('11) have demonstrated
that during states of emotional excitement there is an increase in
the concentration of adrenin in blood drawn from the adrenal
vein of the cat. A similar increase is produced by stimulation of
sensory nerves and by asphyxia (Cannon and Hoskins, '11),
conditions which will be shown to produce a contraction of the
pigment of the horned toad. It is well known that adrenin
occupies an especially significant place in the physiology of smooth
muscle. Langley ('01), Elliott ('05), and others have shown that
the action of adrenin upon this tissue in a great number of cases
is identical with the effect of stimulating the sympathetic fibers
innervating the smooth muscles of the body. Spaeth ('16a)
has concluded that melanophores ''are to be considered function-
ally modified smooth muscle cells.'' He has accumulated evi-
dence which shows at least that there is a very close analogy
between the two types of cell.

In view of these facts, it would not be surprising to find that
adrenin is the hormone concerned in coördinating the melano-
phores of the horned toad.

It has been pointed out by Coronae Moroni ('98), and by Lieben
('06) that adrenin produces a contraction of the melanophore pig-

ment of the frog. Stockard ('15) and Spaeth ('16a) report the same to be true of the melanophores of Fundulus.

If a dilute solution of adrenin[6] in isotonic sodium chloride solution is injected under the skin of a horned toad, a complete contraction of the pigment ensues; the dark bands across the back and legs stand out boldly against the ground-color, which becomes bright cinnamon-buff (fig. 12). This condition is produced by injections of 0.2 cc. of a solution of adrenin diluted to one part in 100,000 and occasionally by a solution of one part in 1,000,000. Weaker solutions, 1 : 10,000,000 and occasionally 1 : 100,000,000, produce a contraction of the melanophore pigment over a more circumscribed area, centering about the point at which the solution is introduced (fig. 11). The latter effect is especially interesting because it is a close duplication of the condition produced by injecting blood from an excited horned toad into an unexcited individual. A comparison of figures 10 and 11 will make this point obvious.

It may be concluded from these facts that adrenin produces an effect upon the melanophores of the horned toad identical with the action of the hormone which appears in the circulation of these lizards during nervous excitement.[7]

The adrenal glands of the horned toad consists of two elongated, yellow bodies situated in the membranes which support

[6] 'Adrenalin-chloride' of a concentration of 1 : 1000 in physiological salt solution prepared by Parke-Davis and Company has been employed as a starting-point in making up the solutions used throughout these experiments.

[7] Gudernatsch ('14) has reported that tadpoles of Rana temporaria "fed on (horse) adrenal cortex become much lighter than those fed on adrenal medulla or any other food."

This observation suggests that some secretion of the cortical part of the adrenal gland may be the melanophore hormone, instead of adrenin itself. This hypothesis was investigated in the following way. Extracts of the cortex and of the medulla of fresh beef adrenals were made. It was found that both extracts produced a contraction of the melanophores of Fundulus heteroclitus and of the horned toad. *The extract of the medulla was effective at much greater dilutions than that of the cortex.* Upon examining these extracts chemically it was found that both contained adrenin, but the extract of the medulla contained a much greater quantity than did the cortical extract. It seems justifiable, therefore, to conclude that adrenin is the substance concerned in the contraction of the melanophores, and not some constituent of the cortical part of the adrenal gland.

the gonads. When they are fixed in a mixture of formol and Müller's fluid and sectioned, it is found that the peripheral portion is composed of alveolar masses of tissue which take on the yellow stain characteristic of the medullary substance of the mammalian adrenal gland. Extracts made by grinding a pair of these glands in 2 cc. of physiological salt solution cause an inhibition of the tonic contractions of the intestine of the white rat (figure C). This reaction is characteristic of adrenin (figure B) Extracts made in a similar way from other tissues of the horned
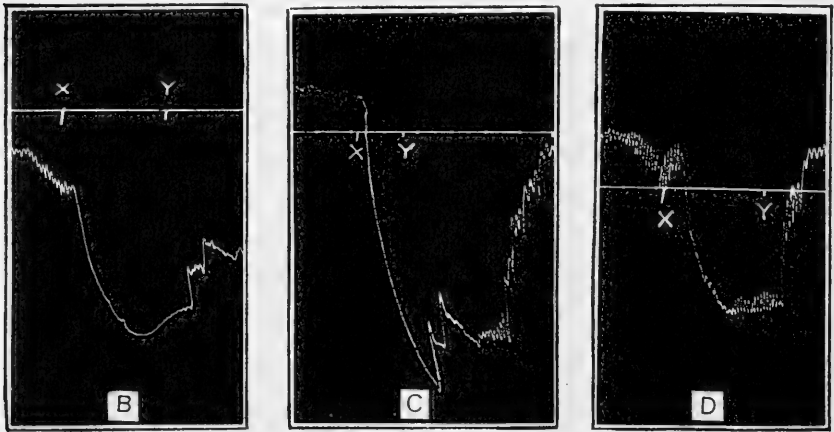
Fig. B Record of the effect of adrenin upon the rhythmic contractions of the intestine of the rat. At **X** adrenalin chloride 1 : 100,000 in Ringer's solution was introduced. At **Y** Ringer's solution replaced the adrenalin solution.

Fig. C Record of the effect of an extract of the adrenal glands of the horned toad (introduced at **X**) upon the rhythmic contractions of the intestine of the rat. At **Y** Ringer's solution replaced the adrenal extract.

Fig. D Record of the effect of an extract of the epididymis of the horned toad (introduced at **X**) upon the rhythmic contractions of the intestine of the rat. At **Y** Ringer's solution replaced the extract.

toad, such as the liver (figure E), skeletal muscle (figure F), and testis (figure G), do not produce this reaction. Extracts prepared from the epididymis do cause an inhibition of intestinal muscle (figure D), but this action is readily understood when it is considered that the adrenal glands lie in immediate juxtaposition

to the epididymis and that a small portion of adrenal tissue may readily have been incorporated in the extract.

When extracts made from the adrenal glands of the horned toad are injected under the skin of living specimens of this lizard, a contraction of the melanophore pigment is produced.   Extracts of liver, of skeletal muscle, and of testis do not cause any contraction of the pigment.   Extracts of the epididymis produce a contraction of the melanophore pigment of horned toads into which they are injected, but, as pointed out, these extracts have been shown to contain adrenin.
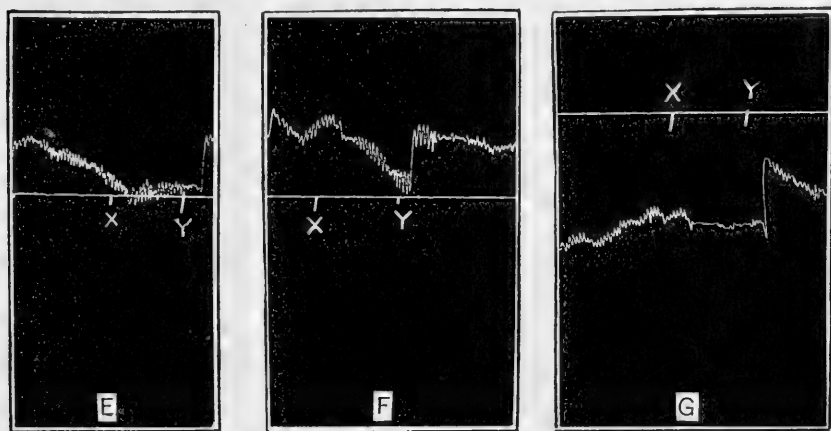
Fig. *E*   Record of the effect of an extract of the liver of the horned toad (introduced at **X**) upon the rhythmic contractions of the intestine of the rat.   At **Y** Ringer's solution replaced the extract.

Fig. *F*   Record of the effect of an extract of the skeletal muscle of the horned toad (introduced at **X**) upon the rhythmic contractions of the intestine of the rat.   At **Y** Ringer's solution replaced the extract.

Fig. *G*   Record of the effect of an extract of the testis of the horned toad (introduced at **X**) upon the rhythmic contractions of the intestine of the rat. At **Y** Ringer's solution replaced the extract.

From the foregoing it appears that the adrenal glands of the horned toad contain a substance, adrenin, which is capable of producing a contraction of the melanophore pigment of this animal.   The question is thus raised: can the adrenal glands,

during functional activity, produce enough of this hormone to cause a contraction of the melanophore pigment in the skin?

A difficulty arises in attempting to discover the answer to this question. In order to stimulate the adrenal glands, the body cavity must be opened. If the animal is etherized before performing this operation, the melanophore pigment contracts and renders the continuance of the experiment futile. If eitherization were omitted, the operation of itself would constitute a noxious stimulus and produce a contraction of the pigment. Fortunately, it has been discovered that the destruction of the spinal cord between the eighth and thirteenth vertebra prevents the contraction of the melanophore pigment which normally follows noxious stimulation. After operating on horned toads in this way, it is possible to open the body cavity without causing the melanophore pigment to contract. The use of anaesthetics is rendered unnecessary because the destruction of the cord prevents the passage of nervous impulses from the posterior portion of the body to the brain.

If the adrenal glands of such a preparation be stimulated directly by a weak faradic current, a complete contraction of the melanophore pigment will occur in a few minutes. That this result is not due to a nervous reflex is shown by the fact that if a ligature is tied about one of the hind legs before the stimulation is commenced, the leg will remain in its original dark condition after the rest of the body has become pale (fig. 13). Upon removing the ligature, the leg will become as pale as its mate, though stimulation has ceased several minutes before.

If a ligature is tied about the blood-vessels which supply the adrenal gland, no change in the condition of the pigment cells can be detected upon stimulating the adrenal gland until after this ligature is removed.

From these experiments it becomes quite clear that the adrenal glands of the horned toad can produce a hormone when they are brought into functional activity which contracts the melanophore pigment. The coloration produced in this way resembles in every respect that occurring during nervous excitement.

If adrenin is the hormone responsible for the contraction of the melanophore pigment following noxious stimulation, it should be possible to recognize its presence in the circulation at such a time by its characteristic effects upon other bodily conditions. The action of adrenin upon the heart, blood pressure, alimentary musculature, and the iris do not form good criteria of the presence of adrenin in the circulation during emotional states, because in such a small animal as the horned it is very difficult to be sure the effects are not produced through the sympathetic nervous system.

The presence of adrenin in the circulation, however, causes a change in its sugar content. It has been shown by Paton ('03), Bierry et Gatin-Gruzewska ('05), and others that glucose appears in abnormally large quantities in the blood stream after the injection of adrenin into it. Blum ('01) has discovered that adrenin injections will produce glycosuria, another manifestation of the same action of this hormone. Cannon, Shohl and Wright ('11) found that when cats are excited for even so short a time as one-half hour they exhibit glycosuria. This emotional glycosuria is produced by the secretion of adrenin which accompanies emotional excitement. If adrenin occurs in abnormal quantities in the circulation of the horned toad during states of excitement, its presence should be indicated by an increase in the concentration of blood sugar.

The sugar content of the blood of fifteen horned toads was determined by the method of Myers and Bailey ('16). The first series, representing the normal sugar content of the blood in five individuals, was made by drawing blood from animals which were unexcited and in which the melanophore pigment was fully expanded. The second series represents the sugar content of the blood of five horned toads which were thrown into a state of nervous excitement by stimulating the mouth with a weak induction current for thirty minutes and then waiting thirty minutes before drawing the samples of blood. The third series represents the sugar content of the blood of five horned toads into which 0.5 cc. of a 1 : 1000 solution of adrenin had been injected one hour before. Table 1 shows the result of this experiment.

TABLE 1

Per cent of sugar in blood of horned toads

NORMAL	EXCITED	INJECTED WITH ADRENIN
0.09	0.13	0.10
0.12	0.16	0.21
0.14	0.17	0.23
0.15	0.18	0.34
0.15	0.20	0.44
Mean 0.13 ± 0.01	Mean 0.17 ± 0.01	Mean 0.26 ± 0.04

It is clear that the sugar content of the blood in those animals which had undergone nervous excitement is higher than in the normal animals, but not as high as in animals into which adrenin has been injected. The conclusion is warranted that the horned toad exhibits an emotional hyperglycemia, which indicates that adrenin is secreted into the blood stream during nervous excitement.

Although a number of very sensitive tests for adrenin are known, no one, according to Stewart ('12), has succeeded in demonstrating the presence of adrenin in the general circulation of mammals. In the hope that the adrenal secretion of the horned toad might be greater than that of mammals, an attempt has been made to detect adrenin in the blood of these animals during states of nervous excitement. The method devised by Cannon and de la Paz ('11) was finally selected for this purpose. This test depends upon the inhibition of the rhythmic contractions of the longitudinal muscles of the intestine by minute quantities of adrenin. The method was successfully modified so that adrenin could be detected at dilutions of one part in ten million, and in quantities of blood so small as 0.7 cc. Although a number of positive tests for adrenin were obtained from blood from excited horned toads, the majority of the results were so variable that no weight can be placed upon the experiments. The difficulty which must attend the detection of adrenin in the circulation prevents this negative evidence from having any effect upon the hypothesis developed in this paper.

If adrenin is produced in the circulation of the horned toad during nervous excitement and is responsible for the contraction of

the melanophore pigment at this time, one might expect to find the pigment contracted under other conditions which are known to activate the adrenal glands.

Cannon and Hoskins ('11) found that during asphyxiation the adrenal glands of the cat are activated. Itami ('12) suggests that the effect of carbon dioxide upon the vascular system is probably due in part to an increased secretion of adrenin. If horned toads are placed in a glass vessel through which carbon dioxide is passed, the skin becomes very pale in ten minutes. At this time the animals have become lethargic, but are still able to recover from the treatment.

Etherization decreases the residual adrenin in the cat's adrenal glands about 50 per cent, according to Elliott ('12). The melanophores of horned toads almost invariably exhibit a contraction of their pigment when the animals are etherized.

Morphia has been shown by Elliott ('12) to cause exhaustion of the adrenal glands. If 0.1 cc. of a 10 per cent solution of morphia oleate is injected under the skin of a horned toad, the pigment becomes contracted completely within a few minutes.

Nicotine has an effect upon the adrenal glands similar to that of morphia (Cannon, Aub and Binger, '16). Injection of nicotine also produces a contraction of the melanophore pigment of the horned toad. This effect is due in part to the direct action of this drug upon the pigment cell, for if nicotine is injected into a leg about which a ligature is firmly tied, this leg alone will become pale.

It is quite possible that the effects of asphyxiation, etherization, and morphia are also due to a direct action upon the pigment cells. At least it can be said that there is no contradiction between the observed behavior of the pigment cells and the behavior to be expected if they are affected by the secretion of the adrenal glands. The melanophore pigment of the horned toad is contracted during nervous excitement, asphyxia, ether anaesthesia, and poisoning by morphia and nicotine—conditions which are known to produce activity of the adrenal glands in mammals.

If it could be shown that changes occurred in the histological condition of the adrenal glands of the horned toad or that these bodies become exhausted as the result of any stimulus which will cause a contraction of the pigment, additional evidence that the melanophores are coördinated by the secretion of adrenin would be afforded. It has not been possible to produce any recognizable change in the condition of the glands either by prolonged noxious stimulation or by stimulating the glands directly with an electric current. The method employed, fixing with a mixture of formol and Müller's fluid, evidently was not suitable for detecting any changes which may occur in the adrenal glands of these lizards. Elliott ('12) was able to demonstrate only a very slight decrease in the residual adrenin after faradizing one of the splanchnic nerves of the cat.

Although the preceding experiment failed to demonstrate any exhaustion of the adrenal glands, an observation has been made which is significant in this connection. When shipments of horned toads first arrive from Texas the skin of the animals is always very dark in color. If these lizards are subjected to a noxious stimulus, the melanophore pigment becomes contracted only slightly and much more slowly than normally. Some individuals fail to exhibit the reaction at all. Not until after a week does the reaction become normal. When it is remembered that these animals have been crowded together in a small box, and shaken about in an express car for at least five days, it is not surprising that any gland which is brought into activity by nervous excitement should become exhausted. The failure of the reaction in these animals may be explained readily in this way. The bearing of this observation upon the relation of the adrenal glands to the reaction of the melanophores to noxious stimuli will become evident when it is pointed out that Elliott ('12) has found that the adrenin content of the adrenal glands of cats which have been recently brought into the laboratory is below that of animals which have become accustomed to their surroundings.

If the contraction of the melanophore pigment of the horned toad which follows noxious stimulation is due to a secretion of the

adrenal glands, removal of these bodies might be expected to check the reaction. It is not difficult to open the body cavity of a horned toad, tie ligatures about the membranes supporting the adrenal glands, and cut out these bodies. It is necessary to take with them the gonads and a portion of the genital ducts. Although the operation involves tying off the postcava, other veins are able to compensate for the loss of this vessel, and the animals live for a week or more. It was stated in a preliminary communication (Redfield, '16) that this operation does not check the contraction of the melanophores by noxious stimuli. This observation was based upon experiments upon only a few animals. A larger series has since been operated upon and two horned toads found which fulfilled the requirements of the hypothesis. Although these animals became very pale in less than four minutes when the mouth was stimulated by a weak faradic current of electricity, *after the removal of the adrenal glands no contraction of the melanophore pigment resulted, although the mouth was stimulated in the same way for twenty minutes.* The conclusion is unavoidable that the hormone responsible for the original contraction of the melanophore pigment, as the result of noxious stimuli, is produced by the adrenal glands.

Turning to the larger number of animals in which removal of the adrenal glands proved ineffective in checking the reaction, it must be pointed out that in these horned toads the contraction of the melanophore pigment was somewhat slower and the skin never became as pale as before adrenalectomy. On page 308 an experiment will be described which proves conclusively that this incomplete reaction is due to the action of the nervous system upon the melanophores and shows that when the nervous system has been destroyed removal of the adrenal glands is necessary before the reaction can be blocked.

In the foregoing pages evidence has been presented indicating that: 1) adrenin contracts the melanophore pigment of the horned toad; 2) the adrenal glands contain this substance; 3) stimulation o᷍ these glands causes the melanophore pigment to contract; 4) following noxious stimulation, adrenin occurs in the circulation and the melanophore pigment contracts; 5) the

melanophore pigment is contracted at other times when he presence of adrenin in the circulation is to be expected, and 6) the removal of the adrenal gland hinders or prevents the contraction of the melanophore pigment by noxious stimuli.   In view of these facts, it must be concluded that the hormone which causes the melanophore pigment of the horned toad to contract during states of nervous excitement is adrenin, the active principle of the adrenal glands.

2. Coördinative action of the nervous system

a. The nervous control of melanophores. Any experiments designed to demonstrate the innervation of pigment cells are invalid unless care is taken to eliminate the action of hormones upon the part of the body under examination.   The production of adrenin in response to noxious stimuli presents a real difficulty in such an investigation because the operative procedure involved in cutting and stimulating nerves is quite sufficient to produce a contraction of all the melanophore pigment through the action of this hormone.   This difficulty has been overcome by destroying the anterior part of the spinal cord of the horned toad to be experimented upon.   When this is done the animals recover completely, but no longer respond to noxious stimuli by contracting the melanophore pigment.   Apparently the operation destroys the 'center' or the tracts of efferent nerve fibers which activate the adrenal glands.   The operation has the additional advantage of allowing one subsequently to operate on the posterior part of the body without the use of anaesthetics.

The following experiment shows the effect of stimulating the spinal and sciatic nerves of horned toads prepared in this manner:

August. 11, 1915. Etherized two horned toads and removed the spinal cord between approximately the eighth and the thirteenth vertebrae.

August 12, 1915. Skin of right side of back of one of these animals was cut open and a spinal nerve (the twelfth?) dissected out and stimulated with a weak faradic current for ten minutes.   Produced absolutely no change in the melanophores of any part of the skin.

Stimulated the sciatic nerve of the left hind leg of the other animal for five minutes with a weak induction current.   Produced a very

clear contraction of the melanophore pigment of the left leg, which brought out in sharp contrast the dark and light bands (fig. 14).

These experiments have been repeated many times. In no case has stimulation of the spinal nerves produced any contraction of the melanophore pigment. The results of stimulating the sciatic nerve are extremely variable; often no contraction of the pigment is produced. In many individuals, however, stimulation of fibers which occur in the sciatic nerve produced a contraction of the melanophore pigment.

Many attempts have been made to isolate various portions of the skin from the central nervous system by cutting through the nerves supplying these parts. The sciatic nerve has been cut in the thigh, groups of spinal nerves have been severed close to the spinal cord, and cuts have been made through the body wall so as to isolate completely portions of the skin in the manner described on page 288. In no case have these operations altered the reactions of the melanophores of the isolated regions in any way. The responses of the melanophores to direct stimulation and to hormones evidently suffice to bring about all ordinary melanophore reactions without the aid of nerves which connect with these cells directly.

One operation of this type has yielded a pertinent observation. When the spinal cord of a horned toad is transected at about the level of the thirteenth vertebra, the melanophore reactions are ordinarily unaffected in every way, except that a noxious stimulus applied to the posterior part of the body no longer produces a contraction of the melanophore pigment. Under usual laboratory conditions, the skin of such animals remains in a uniform dark condition. Three cases have been found to which the foregoing statement does not apply. On the day following the operation these horned toads had recovered fully, but the condition of the melanophores was exceptional. On the anterior half of the back the melanophore pigment was completely contracted, giving this portion of the skin the maximum pale condition. Posterior to the point at which the cord had been sectioned, the melanophore pigment remained fully expanded. The skin of this portion was quite dark (fig. 15).

Why the anterior portion of these animals became pale is not obvious. The effect cannot be due to a secretion of adrenin or any other hormone, nor to the direct action of any environmental factors, since these would affect all parts of the skin uniformly. The coincidence of the point of operation upon the spinal cord with the line of demarkation between the dark and pale areas of the skin point to a causal connection between the two. The conclusion is thus suggested that the posterior part of the body is isolated by transection of the spinal cord from some nervous disturbance which is acting upon the melanophores of the anterior part of the body through nerves connecting directly with these cells. These observations, although exceptional, lend strong support to the conclusion drawn from the contraction of the melanophore pigment of the leg by stimulation of the sciatic nerve; the melanophores are acted upon directly by nerves, and their pigment is contracted by nervous impulses.

That the preceding interpretation is correct is shown clearly by an experiment which demonstrates, in addition, the relation existing between the adrenal glands, the nervous system and the melanophores. It has been pointed out that cutting the nerves distributed to a part of the skin does not interfere with the contraction of the melanophore pigment which follows noxious stimulation. The contraction which occurs at this time has been attributed to the action of the secretion of the adrenal glands upon the melanophores. If the adrenal glands are removed from horned toads which have had the nerves to a portion of the skin transected, the isolated region is no longer affected by noxious stimuli, although the melanophore pigment of the remainder of the skin is made to contract. By this procedure the effect described in the preceding experiment may be exactly duplicated (fig. 16).

August 9, 1916. Stimulated the mouth of a horned toad for five minutes with a weak faradic current. A complete contraction of the melanophore pigment resulted.

Etherized the animal and *transected spinal cord at thirteenth vertebra.*

August 11, 1916. Melanophore pigment was expanded. Stimulated mouth as before for ten minutes. A *uniform* contraction of the melanophore pigment resulted. The operation has not affected the reaction of the pigment cells in any part of the skin.

Etherized the animals and removed the adrenal glands.

August 12, 1916. Melanophore pigment was uniformly expanded. Stimulated mouth as before for ten minures. *The back anterior to the point of transection of the spinal cord,* the fore limbs and all the lateral scales *became very distinctly paler.* The melanophore pigment of posterior part of the body remained fully expanded.

This experiment demonstrates clearly the mechanisms concerned in the coördination of the melanophores. Before removing the adrenal glands the melanophore pigment of a portion of the skin which is isolated from the nervous system may be made to contract; after removing these glands this reaction can no longer be brought about. It is thus unquestionably proved that the secretion of the adrenal glands is one factor in the coördination of the melanophores.

After the adrenal glands have been removed it may also be clearly seen that the nervous system is directly concerned in the coördination of melanophores. It is then possible to cause a contraction of the melanophore pigment of the entire skin, excepting those parts which have been isolated from the rest by the transection of nerves.

It now becomes quite clear why cutting nerves to a part of the skin does not prevent the melanophore pigment of that part from contracting when the animal becomes excited, and why removing the adrenal glands usually does not block this reaction completely. *The melanophores are coördinated by two distinct mechanisms, the adrenal secretion and the direct action of nerves. Either mechanism alone is capable of causing the melanophore pigment to contract.* It is consequently necessary to isolate the melanophores from both factors before their reactions are blocked.

A few horned toads have been found in which removal of the adrenal glands alone has served to block the reaction of the melanophores to noxious stimuli. In other individuals stimulation of the sciatic nerve has failed to cause the melanophore pigment of the leg to contract. These facts suggest that in these individuals the nervous control of the pigment cells was not fully developed. In animals from which the adrenal glands have been removed the contraction of the melanophore pigment is never as great as that produced by the combined action of the adrenal

secretion and direct nervous action. It may be concluded, therefore, that the nervous control of the melanophores is of secondary importance to the coördinative action of adrenin upon these cells.

b. The nervous paths of melanophore reflexes. The nervous system may be explored readily in search for the paths over which the impulses induced by noxious stimuli travel to the melanophores, by cutting away various parts of it and then attempting to produce a contraction of the melanophore pigment by stimulating the animal.

Decerebration does not interfere with the reaction of melanophores to noxious stimuli. After the entire brain anterior to the cerebellum has been cut away, horned toads recover and may live many days. The melanophore pigment becomes expanded, but may be caused to contract by stimulating the surface of the cloaca with an induced current of electricity. If the spinal cord is sectioned at the level of the second vertebra, stimulation of the cloaca may still produce a contraction of the melanophore pigment. It is evident that the medulla and the anterior part of the cord are unnecessary for the reaction. If the cord is cut at the level of the twelfth vertebra, or destroyed completely posterior to this level, stimulation of the mouth by a weak induction current will produce a distinct contraction of the melanophore pigment. The posterior portion of the cord is thus unessential for the completion of the reflex. There remains the portion of the spinal cord which lies between the second and twelfth vertebrae, part of which appears to be necessary for the contraction of the melanophore pigment which follows a noxious stimulus. It has been pointed out in another place (p. 300) that if the cord is destroyed between the eighth and twelfth vertebrae noxious stimuli are no longer able to bring about this reaction. The portion of the cord between the second and eighth vertebrae is not capable of bringing about a contraction of the melanophore pigment. It appears that in the region between the eighth and thirteenth vertebrae are located nervous structures, perhaps 'nerve centers,' through which those nerve impulses pass which cause a contraction of the melanophore pigment.

Nervous impulses reach the part of the cord in question from either end. In the case of noxious stimuli they no doubt travel over sensory nerves. Impulses owing their origin to the color of the environmental substratum probably travel over the optic nerves and thence posteriorly through the brain and spinal cord.

Nervous impulses pass out from this region either to the adrenal glands or to efferent nerves which connect directly with the melanophores. Since the experiments cited above indicate that the portion of the spinal cord anterior to the eighth vertebra cannot produce a reaction of the melanophores, and the portion posterior to the thirteenth vertebra is unnecessary for a reaction, those impulses which pass to the adrenal glands must traverse nerves which leave the cord between the eighth and thirteenth vertebrae.

The course of those impulses which pass directly to the melanophores is indicated roughly by the experiment described on page 308. Since it is possible to isolate the posterior portion of the skin, from these impulses by simply cutting the spinal cord, it may be concluded that the nerve tracts which carry impulses to the melanophores run posteriorly, and probably anteriorly, within the spinal cord to the segments of the body in which the affected melanophores lie. From these points they leave the cord to traverse segmental peripheral nerve-trunks to the melanophores.

What class of nerve fibers carry these impulses along the peripheral nerves? The fact that the melanophores are coördinated through the activity of adrenin indicates that the sympathetic division of the automonic nervous system is involved. After comparing the action of sympathetic nerves and that of adrenin upon a long list of organs, Elliott ('05, p. 466) states: "A positive reaction to adrenalin is a trustworthy proof of the existence and nature of sympathetic nerves in any organ." Spaeth ('16a) has shown that there is a very close analogy between the physiological behavior of melanophores and smooth muscle, a tissue which is almost invariably innervated by the sympathetic division of the autonomic nervous system.

Summary

1. The melanophore pigment of the horned toad is contracted by the direct action of nerves as well as by the action of adrenin.

2. The spinal cord contains, between the eighth and thirteenth vertebrae, nervous structures through which pass the impulses which cause the contraction of the melanophore pigment.

3. Impulses pass from this part of the cord directly to the adrenal glands.

4. Impulses also pass from this part of the.cord posteriorly, and perhaps anteriorily, within the cord to segmentally arranged peripheral nerves which connect directly with the melanophores.

5. The peripheral fibers are a part of the sympathetic division of the autonomic nervous system.

VI. DISCUSSION

1. The nature of the contraction of melanophore pigment

Before attempting to make a unified presentation of the mechanism of color change in the horned toad, it will be valuable to examine the nature of the changes occuring in the melanophore which bring about a migration of the pigment granules. The most recent consideration of this subject is by Spaeth, who says ('16a p. 210):

> Considered as a physical-chemical system the melanophore consists essentially of a colloidal suspension of melanin granules. . . . . When the melanophores are stimulated to contract, we observe the first step in a reversible aggregation or coagulation process, i.e., the aggregation of the melanin granules. Is it possible to consider this phenomenon a reversible coagulation such as occurs commonly in emulsoids?

This question is answered in the affirmative. Spaeth concludes in addition (p. 213) that

> In the contraction of the melanophore there is an aggregation of melanin granules which is to be considered the visible counterpart of an aggregation of colloidal particles that occurs during the contraction in smooth, and possibly striated muscle.

In attempting to elucidate the nature of tonus in muscle, Sherrington ('15, pp. 229 to 230) presents a conception which harmonizes well with the conclusion of Spaeth:

The facts tend to show that in many cases postural contraction (as Sherrington designates tonus) is astonishingly economically maintained; that the turnover of chemical energy involved by it is extremely low. In such cases its relative unfatiguability may well be related to its economy of maintenance. So strikingly does this aspect of it contrast with the expense of maintenance and the relatively rapid fatiguability of ordinary tetanic contraction as to suggest that the chemico-physical process underlying postural contraction is in part at least essentially other than that underlying twitch contraction, and the fusion of twitch contractions termed tetanic contraction.

The supposition has been put forward that in maintaining this economical postural contraction the muscle-fiber or some part of it, clots, changes from sol to gel. . . . . On this view evidently one of the nerves . . . . . can cause the contents of its muscle-fibers to gel, to solidify, another of its nerves cause it to unclot.

Such a view is easily applicable to visceral muscle such as vesical and gastric, and to that of the blood-vessel wall.

It appears, therefore, that there is a marked similarity between the contraction and expansion of melanophore pigment and the tonic changes in the state of smooth muscle. That the contraction of the melanophore pigment resembles the prolonged tonic contraction of smooth muscle more closely than the momentary contraction of skeletal muscle is demonstrated by the fact that the skin of the horned toad will remain pale for months if the animals are kept in a light colored environment. There is no evidence of any fatigue of the melanophore from prolonged contraction, or expansion, of its pigment. Spaeth ('16c) has made a similar observation upon the melanophores of Fundulus.

Spaeth has shown that the state of aggregation of melanin granules within the pigment cell may be altered by a variety of chemical and physical stimuli as well as by the physiological action of nerve impulses. Antagonisms are demonstrated between not only chemical substances, e.g., sodium and potassium, but between chemical and physical stimuli, e.g., atropine and heat (Spaeth, '16b). The tonic state of the melanophore, that is the degree of expansion or contraction of its pigment, may be conceived to depend upon the resultant effect of the various conditions which exist within and about the cell upon its state of colloidal aggregation. Any change in these conditions will upset the equilibrium which has existed; a new point of equilibrium will

be reached and the melanophore pigment will receive a different distribution within the cell. In other words, by changing the balance of factors which affect the melanophore, its tone is changed.

The view that reaction of the melanophores of vertebrates is of a tonic nature has been held by Lister ('58), Pouchet ('76), von Frisch ('11), Krukenberg ('80), Keller ('95), and others. These authors had in mind, however, a tonus established and maintained by the central nervous system, and although it was believed that this tonus might be altered by certain stimuli acting upon the melanophores, the conception was quite different from the view here presented, that the tonus of the melanophores is an inherent quality of these cells.

If this view of the nature of the melanophore be taken, it is obviously futile to speak of the 'active state' of the cell, a conception which has doubtless arisen from comparing the melanophore's reaction with the 'twitch' phenomenon of muscle, rather than the 'tonus' phenomenon. The contracted condition is no more active than the expanded, or at least there is no experimental evidence to that effect. Activity is involved only in changing from one state to the other and in either direction. One may better speak of states of increased and decreased tone in describing the conditions of melanophores. Since Spaeth ('16a) has shown that many of the physiological reagents which produce a contraction of smooth muscle also produce a contraction of the melanophore pigment, the contracted state may be called arbitrarily that of increased tone, the expanded state that of decreased tone in the case of melanophores as well as of smooth muscle.

2. Résumé of the reactions of the melanophores of the horned toad

If the conclusions drawn from the foregoing experiments are correct, the tone of the melanophores of the horned toad is varied in the following ways:

The tonic state of the melanophores is conditioned by the action of nervous impulses, spreading perhaps from a center in he thoracic cord, passing along sympathetic fibers and terminating

either directly in the pigment cells or in the adrenal glands or both. The presence of such impulses delivered directly to the melanophores causes an increase in the tone of these cells and a consequent paling of the skin. Impulses delivered to the adrenal glands cause a secretion of adrenin, which is carried by the circulation to the pigment cells and affects them in a way identical with direct nervous stimulation.

Such nervous impulses may be set up through stimulation of the eyes by the color of the environment. In this way the adaptation of the color of the horned toad to the color of the substratum may be explained. These nervous impulses may also
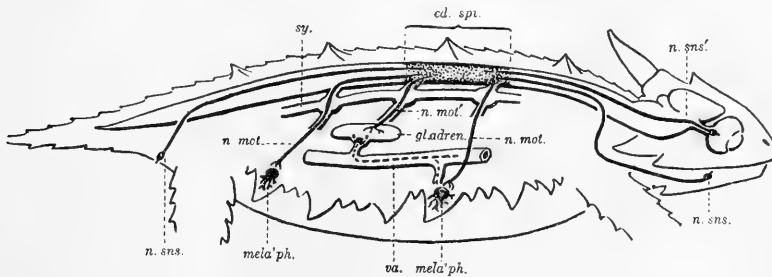
Fig. *H* Diagram of the mechanism coördinating the melanophores of the horned toad. Noxious stimuli are carried along sensory neurones, *n. sns.*, and photic stimuli along other sensory neurones, *n. sns'.*, to a region of the spinal cord between the eighth and thirteenth vertebrae, *cd. spi.* From there impulses pass out along motor neurones, *n. mot'.* to the adrenal glands, *gl. adren.* The secretion of adrenin so induced is carried by the blood-vessels, *va.*, to the melanophores, *mela'ph.* Other impulses travel along other motor neurones, *n. mot.*, which pass through the sympathetic nervous system, *sy.*, directly to the melanophores. The melanophores are affected in addition by illumination and temperature, the action of which is independent of the coördinating systems of the body.

originate from the nervous excitation brought about by noxious stimuli and produce a contraction of the melanophore pigment.

The tonic state established through the action of nerves and the adrenal glands may be varied through the direct action of certain stimuli upon the melanophores or upon tissues closely associated with them. Thus, light and cold tend to decrease the tone so established. Darkness and heat, on the other hand, tend to increase the tone of the pigment cells. By this means the

fluctuations in the state of the pigment cells which make up the daily rhythm are to be explained.

Figure *H* is a schematic representation of the various factors which influence the tone of the melanophores of the horned toad.

3. Comparative physiology of the melanophores of vertebrates

a. *The influence of hormones upon melanophores.* Since many of the more extensive investigations of melanophores were made before the study of the endocrine glands assumed the important place in physiology which it holds to-day, it is not surprising that only one attempt has heretofore been made to associate melanophore reactions with hormones. The suggestion of Fuchs, ('14), that a secretion of the pineal organ may influence melanophore reactions, has been shown on page 295 to be unsupported by the known facts. The only other studies which bear upon the present point are a number of isolated observations that adrenin causes a contraction of melanophore pigment. This observation has been made upon the frog by Corona e Moroni ('98) and by Lieben ('06), and upon Fundulus by Stockard ('15) and by Spaeth ('16a). These authors do not suggest, however, that activity of the adrenal glands is correlated with the condition of the melanophores in nature.

In some unpublished experiments upon Anolis carolinensis, it was found that the melanophore pigment is contracted during nervous excitement, that this contraction could be blocked momentarily in one leg by tying a ligature about it, and that injection of adrenin produced the green color of the skin. It would not be surprising if a more extensive study should show that the conditions found in the horned toad also exist in this lizard.[8]

[8] Since this paper was written an article has appeared by Ruth and Gibson ('17) in which it is stated that the skin of several species of Philippine house lizards becomes blanched through the action of adrenin and mechanical irritation. They consider their results similar to those described in an earlier paper (Redfield 1916). These authors take the novel view that this color change is due to a bleaching of the pigment rather than to a change in the position of the melanin granules in the skin.

Although the foregoing are the only direct observations upon the subject, there are well-known color phenomena in many vertebrates in which the adrenal secretion may play a part.[9] Adrenin is secreted in mammals during emotional excitement. It would not be surprising, therefore, to find the secretion of the adrenal glands responsible for the emotional or 'psychic' color changes of fishes, amphibians, and reptiles which have been frequently described. Since it is recognized that hormones play an important rôle in the development of secondary sexual characters, it is quite possible that the striking color changes which occur in the breeding season or during courtship are, as Fuchs ('06) has suggested, produced by these agents.

b. *The coördination of melanophores and smooth muscle.* There is a certain resemblance between the mechanism coördinating the smooth muscles of mammals and that which coördinates the melanophores of many vertebrates. Both are under the control of the sympathetic nervous system and both are influenced by adrenal secretion during nervous excitement. The smooth muscles, however, are known to be innervated by antagonistic fibers belonging to two morphologically distinct parts of the autonomic nervous system: It may be asked, are the melanophores also under a double innervation?

Bert ('75) suggested that the melanophores of Chameleon are innervated by two sets of nerves analogous to those controlling the blood-vessels. Carnot ('96) and Sollaud ('08) applied a similar explanation to their observations upon the frog. Babák ('10) was forced to conclude that the expansion as well as the contraction of the melanophore pigment of Amblystoma larvae is produced by nervous impulses arising in the retina, because the pigment of tadpoles is expanded in the dark, while that of blinded larvae is contracted in the absence of photic stimuli. Other

[9] It must not be supposed that all vertebrates will be found to exhibit the adrenal control of melanophores to the extent which the horned toad does. One may judge from the experiments performed on the chameleon, the frog, and many fishes that these forms do not possess so sensitive a mechanism, for if they did it would be impossible to execute such experiments as the stimulation of nerves without producing a secretion of the hormone.

investigators do not appear to have pressed the idea of double innervation of the melanophores.

It has been pointed out (Carlton, '03) that the melanophore pigment of Anolis, unlike that of other vertebrates, is expanded by impulses from the autonomic nervous system. This exceptional behavior might be explained in one of two ways. It might be assumed that these melanophores are under the control of the sympathetic division of the autonomic nervous system, and that these nerves affect the melanophores of Anolis by causing the expansion of the pigment instead of the contraction that they cause in other animals. Parallels to this condition are not lacking in mammalian physiology, for the sympathetic division causes a contraction of some smooth muscles (in the blood-vessels) and relaxation of others (in the intestinal walls), as Elliott ('05) and others have abundantly shown. Sympathetic impulses may have antagonistic effects even in the same organ, according to Dale ('06). If such is the condition in Anolis, one might expect adrenin, which mimics the action of the sympathetic division, also to cause an expansion of the melanophore pigment. This, however, is not the case; it is stated on page 316 that adrenin causes the melanophore pigment of Anolis to contract. It seems doubtful, therefore, if the melanophore pigment of Anolis is expanded by impulses from the sympathetic division of the autonomic nervous system.

The alternative explanation is to assume that the melanophore pigment is expanded by impulses from fibers which are physiologically analogous to the *cranial-sacral division* of the autonomic nervous system of mammals. If such be the case, adrenin would be expected to cause the opposite effect upon the melanophores, which indeed it does. This supposition harmonizes with the facts completely. It may further be argued, since adrenin causes a contraction of the melanophores of Anolis, that these cells are also innervated by the sympathetic division of the autonomic nervous system, and that these fibers cause the pigment to contract, for Elliott ('05) has shown that the reaction to adrenin is an excellent indicator of the presence and nature of sympathetic control of a tissue.

From these considerations it may be anticipated that, like the smooth muscles of the mammalian body, the melanophores of Anolis possess a double innervation from the two divisions of the autonomic nervous system.   It may be that such a condition is present quite generally among those vertebrates whose melanophores are under nervous control.   If such a condition exists, it may have escaped detection because the nerve-trunks contain fibers of both sorts, and when they are simulated one sort, usually the sympathetic, dominates over the other in its effect upon the pigment cells.

If the preceding discussion has succeeded in demonstrating the resemblance between the control of melanophores and smooth muscles by the autonomic nervous system and the adrenal glands, it has lent strong support to the hypothesis of Spaeth ('16a) that the melanophores are functionally modified smooth muscle cells.

c. The physiological basis of the emotions.   Cannon ('15) and his collaborators have shown that the mechanism underlying the usual emotional manifestations in man and the other mammals is the autonomic nervous sytsem and its important adjunct, the adrenal glands.   It has now been shown that in the horned toad this same mechanism is brought into play during nervous excitement.   The basis for emotional manifestations appears to be the same in reptiles as in mammals.   The mechanism must be one of great antiquity, for Gaskell ('14) has discovered the location and activity of the chromaffin (adrenal) system in the leech and certain other annelids.   Whether this system is at this time connected in any way with phenomena corresponding to the emotions of the higher animals cannot be stated.

VII. SUMMARY

1. The reactions of the melanophores of the horned toad produce a series of color changes correlated with the rhythm of day and night, an adaptation of the color of the skin to that of the environment, and a characteristic pale condition of the skin during nervous excitement (pp. 278 to 280).

2. The daily rhythm of color change is caused by the direct action of photic and thermal stimuli upon the melanophores or some closely associated tissue (pp. 280 to 282, 284 to 289).

3. The adaptive reactions of the melanophores depend upon stimuli received through the eyes (pp. 290 and 291).

4. The contraction of the melanophore pigment during nervous excitement may be initiated by any noxious stimulus (pp. 279 and 283).   It is brought about by the coöperation of nervous impulses delivered to the pigment cells by the sympathetic nervous system (pp. 306 to 311) and *the secretion of adrenin by the adrenal glands* (pp. 292 to 306).

5. The resemblance between the innervation of melanophores and smooth muscle is indicated (pp. 317 to 319).

More detailed summaries will be found in the text at the end of each section.

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PLATE 1

Fig. 1   The horned toad on the right has become pale after having been kept on a substratum of sand for six weeks.   The other animal, as a result of having been kept upon a substratum of cinders, has retained the dark color shared by both at the beginning of the experiment. $\times \frac{3}{4}$.

Fig. 2   A pair of horned toads, similar to those of figure 1, photographed upon the backgrounds on which they have been kept. $\times \frac{3}{4}$.

1

2
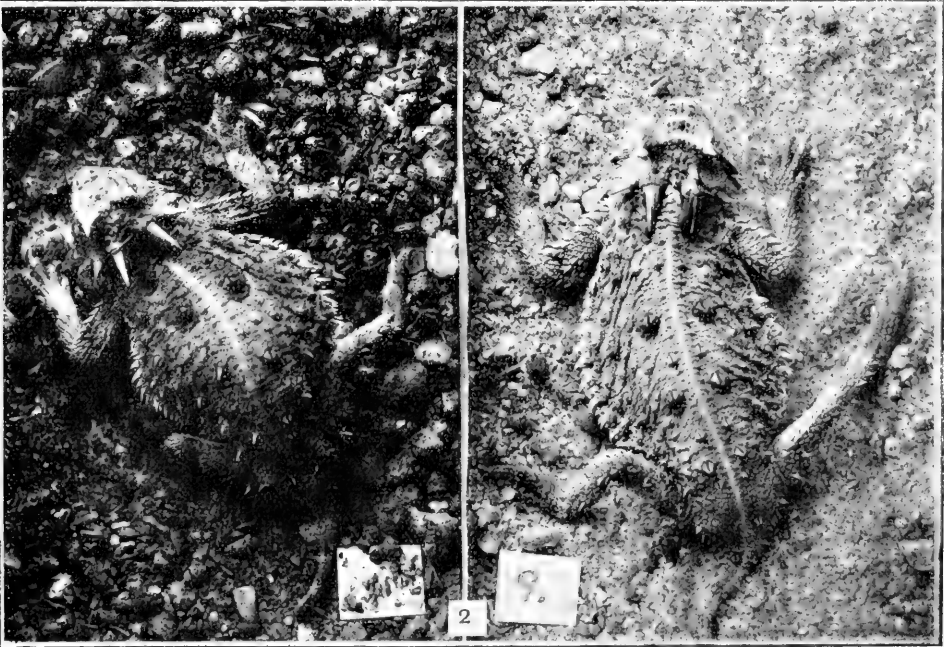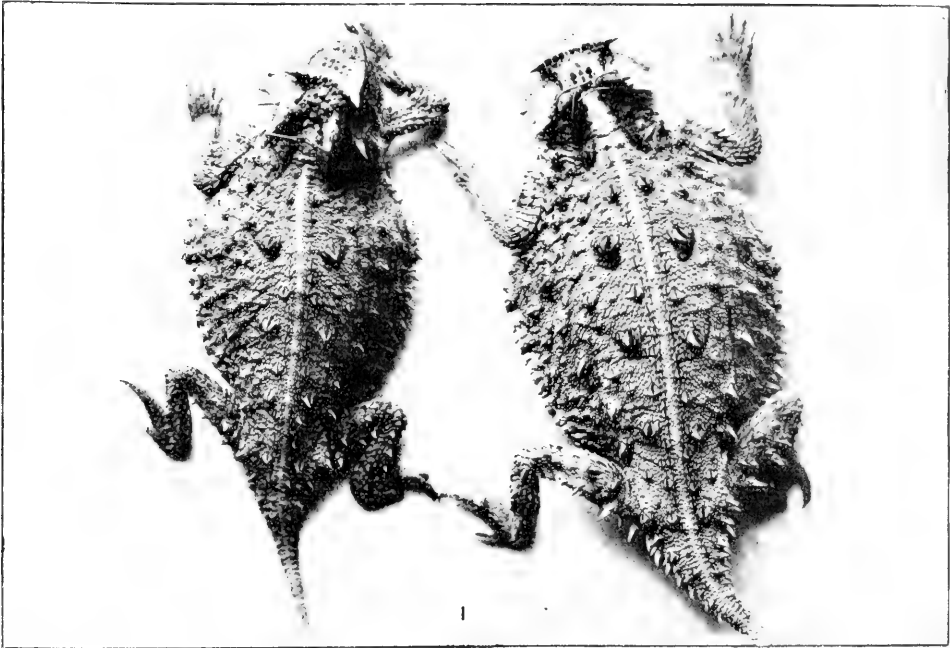
PLATE 2

The horned toads shown in this plate were all equally dark in coloration at the beginning of the experiment, two weeks before the photographs were taken.

EXPLANATION OF FIGURES

Fig. 3   Two horned toads, one of which (*a*) has been kept upon a substratum of cinders for two weeks and has retained its original dark coloration; the other (*b*) has become very pale as the result of being kept for two weeks upon a substratum of white sand.   × ⅔.

Fig. 4   Two horned toads, one of which (*c*) is blindfolded.   In spite of being kept for two weeks upon a substratum of white sand, it has retained its original dark coloration (compare with *a*, fig. 3); the other (*d*) was kept in the same pen with *c*, but having retained its vision, its coloration has become very pale.   × ⅔.

Fig. 5   Two horned toads (*e* and *f*) with a history identical to that of the animals shown, respectively, in *c* and *d*, figure 4.   × ⅔.

Fig. 6   Horned toads which have been kept upon a substratum of sand for two weeks; *g*, although blindfolded in one eye, has become as pale as *h*, which retained complete vision.   × ⅔.

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PLATE 2

MELANOPHORES OF THE HORNED TOAD

ALFRED C. REDFIELD
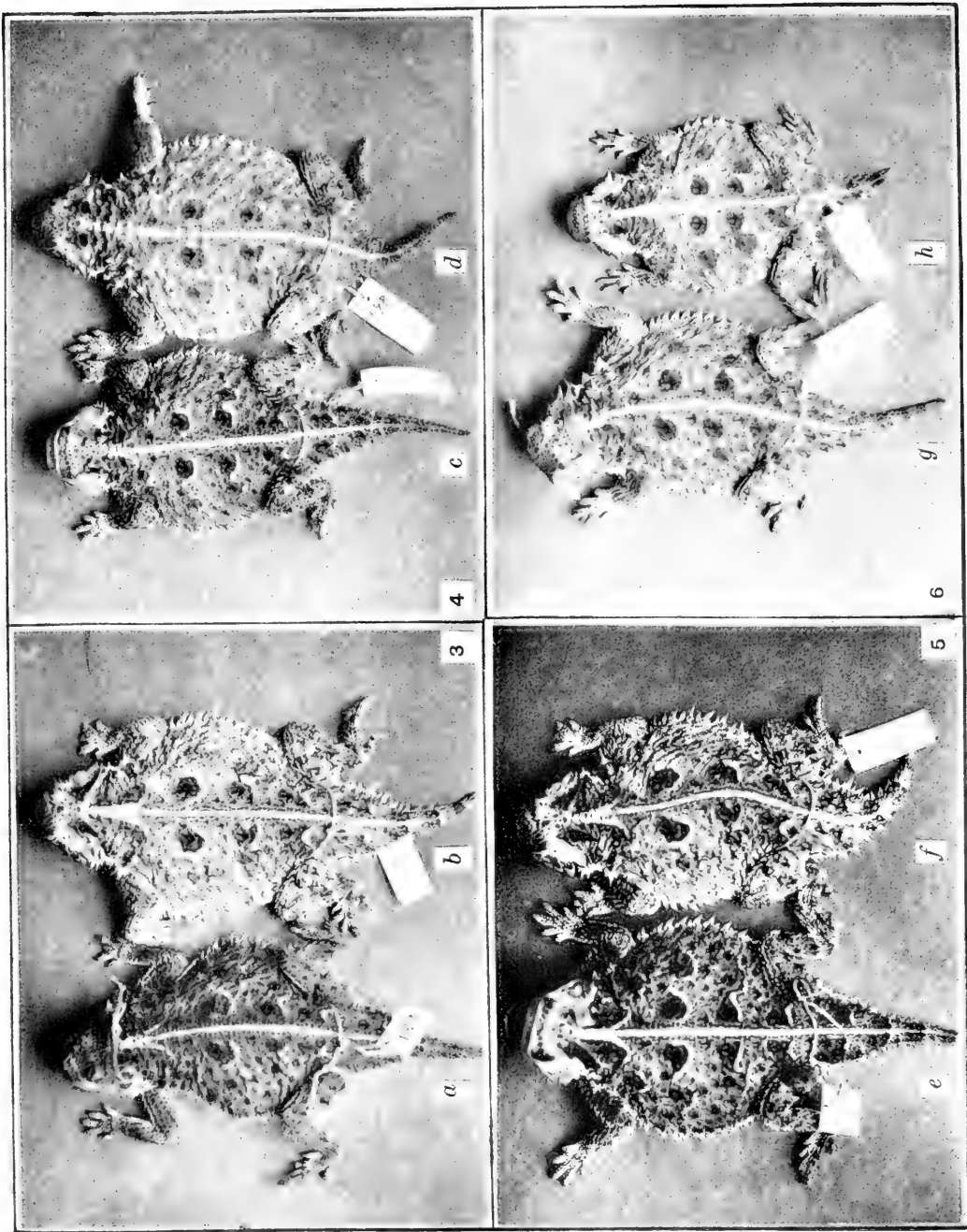
PLATE 3

EXPLANATION OF FIGURES

Fig. 7 The circulation of the right hind leg of this horned toad has been blocked for two and one-half hours. The color of the anemic leg has become paler than that of the remainder of the animal. ×1.

Fig. 8 This horned toad was thrown into a state of nervous excitement after the right hind leg was tightly ligatured. The color of the skin of the entire body has become pale with the exception of the ligatured leg, from which hormones were excluded. The arrow indicates a pale band across the thigh of the left hind leg, which contrasts clearly with the dark coloration of the corresponding part of the opposite leg. ×1.

Fig. 9 The same animal as shown in figure 8, but after removal of the ligature. With the admission of hormones into the right hind leg, it has become indistinguishable from its mate. ×1.

PLATE 3

MELANOPHORES OF THE HORNED TOAD

ALFRED C. REDFIELD

329
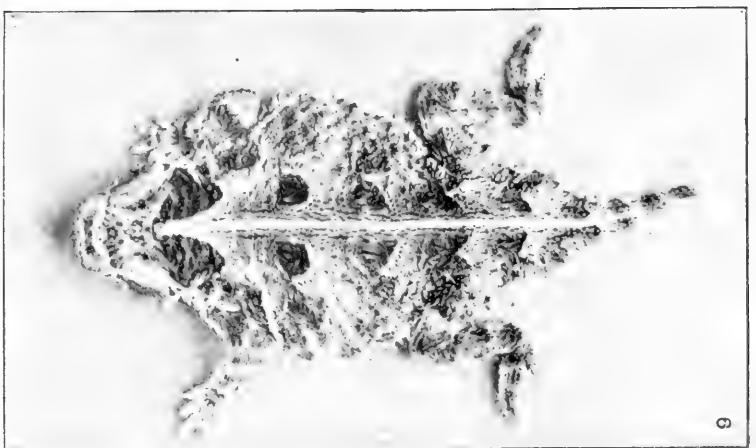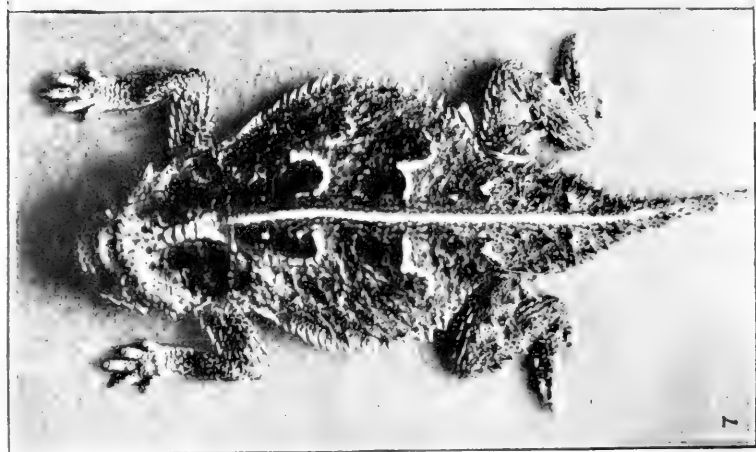
Fig. 10 Blood from an excited horned toad was injected subcutaneously into this animal at the point indicated by the arrow. The skin has become clearly paler around the point of injection. × 1.

Fig. 11 At the point indicated by the arrow, 0.2 cc. of a solution of one part of adrenalin chloride in 10,000,000 parts of 0.75 per cent NaCl was injected under the skin of the horned toad. The skin has become paler at this point, producing an effect identical with that obtained by injecting blood from an excited horned toad into the animal shown in figure 10. × 1.

Fig. 12 Into the horned toad on the left was injected 0.2 cc. of a solution of one part of adrenalin chloride in 100,000 parts of 0.75 per cent NaCl. The pale coloration produced by this means may be contrasted with the usual coloration illustrated by the animal on the right. × 1.

330

10

11

12
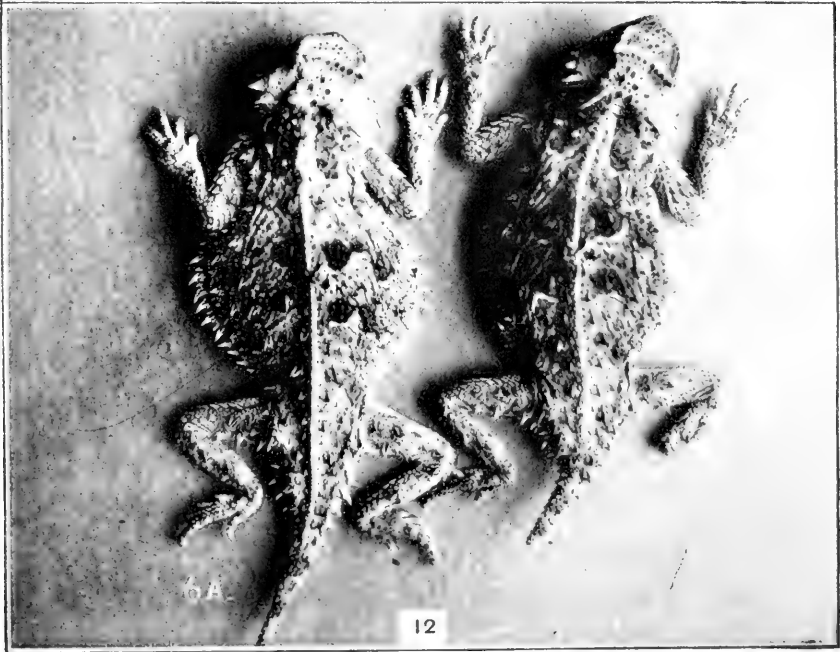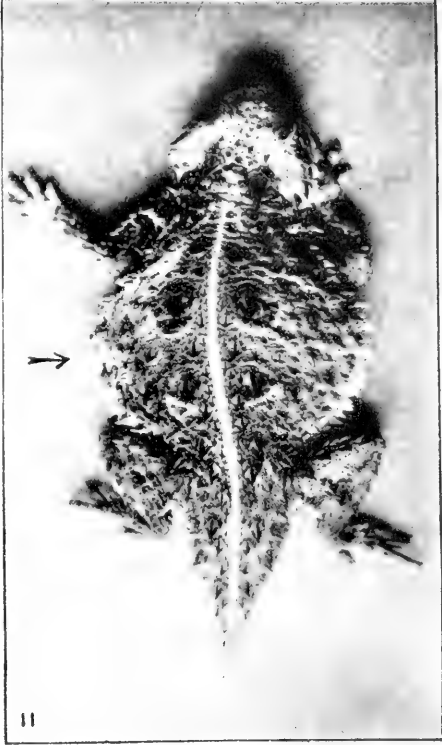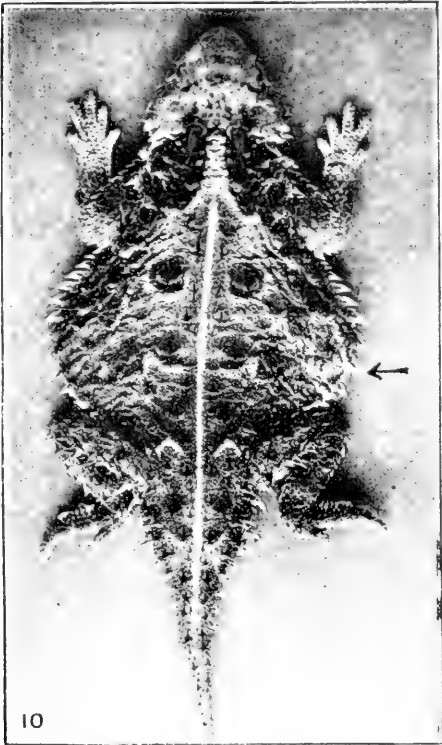
Fig. 13 The adrenal glands of this horned toad were stimulated with a weak faradic current. The melanophore pigment of the entire skin, with the exception of that of the right hind leg, from which adrenin has been excluded by a ligature, has been contracted. Compare with figure 8, in which a similar effect has been produced by excitement. × 1.

Fig. 14 The left sciatic nerve of this horned toad was stimulated at the point indicated by the threads, close to the trunk. As a result the left hind leg is slightly paler than the right. Note particularly the pale patch on the thigh indicated by the arrow. × 1.

Fig. 15 Cutting the spinal cord of this horned toad has caused the posterior half of the skin to remain dark while the anterior half has assumed a pale coloration. The dividing line between the dark and pale areas is indicated by arrows. × 1.

Fig. 16 An effect, similar to that illustrated in figure 15, which was produced in this horned toad only when, in addition to the transection of the spinal cord, the adrenal glands had been removed. × 1.

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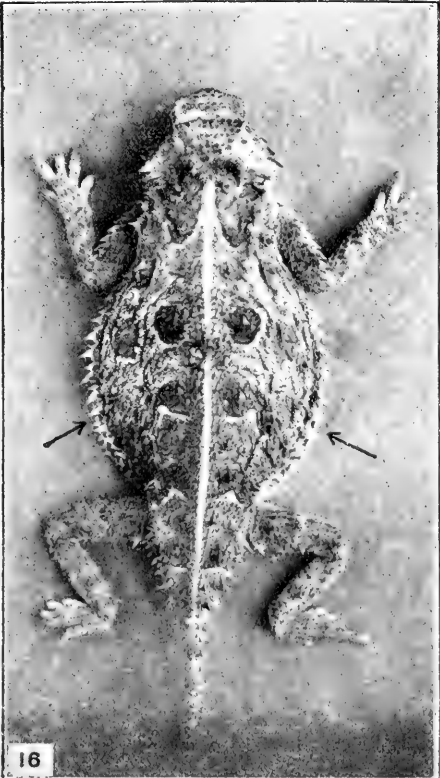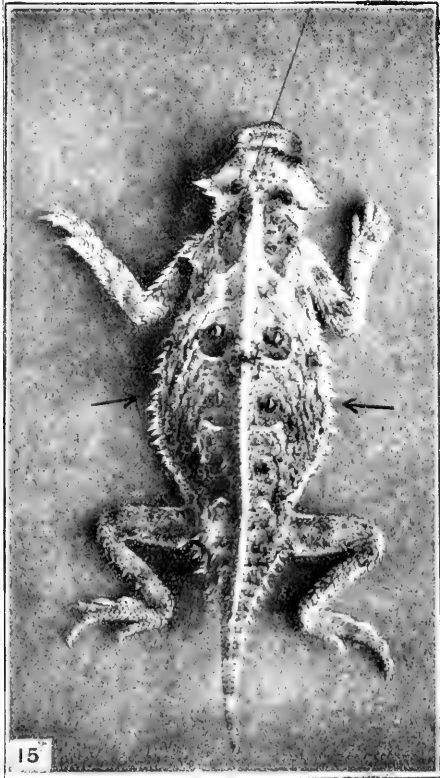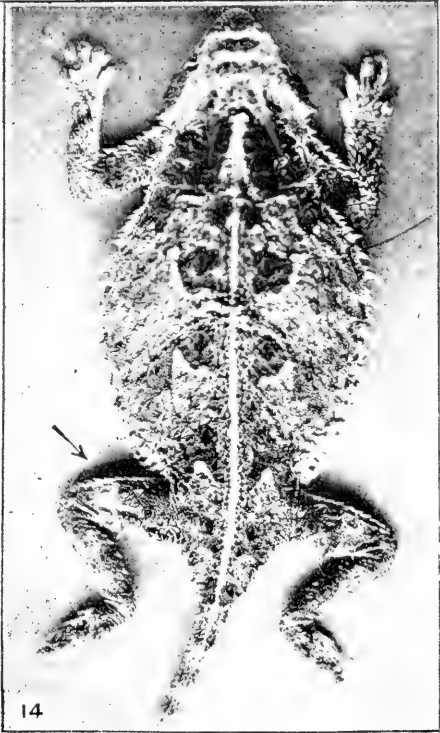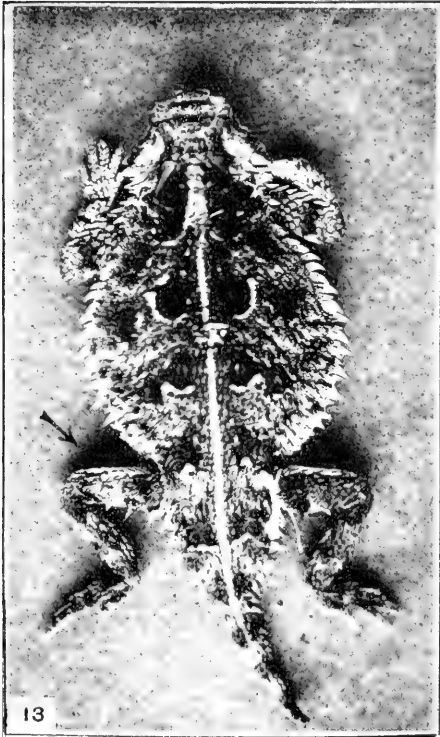
STUDIES ON INBREEDING

II. THE EFFECTS OF INBREEDING ON THE FERTILITY AND ON THE CONSTITUTIONAL VIGOR OF THE ALBINO RAT

HELEN DEAN KING

The Wistar Institute of Anatomy and Biology

TWO CHARTS

The present paper gives data showing the fertility and the constitutional vigor in a strain of albino rats that was inbred, litter brother and sister, for twenty-five successive generations. Details regarding the manner in which these experiments were conducted and data for the growth and variability in the body weight of inbred rats have already been published (King, '18a).

1. THE FERTILITY OF INBRED RATS

As shown by a number of recent investigations (Pearson et al., '99; Rommell and Phillips, '06; Pearl, '12, a, and Wentworth, '16), fertility is undoubtedly a racial character that is transmitted by inheritance, although it is influenced to a considerable extent by a variety of extraneous factors. The mode of inheritance of fertility in the rat is not discussed in the present instance, since the effects of inbreeding on fertility is the chief subject under consideration.

Throughout this paper the word 'fertility' is used as defined by Pearl and Surface ('02) to designate: "The total actual reproductive capacity of pairs of organisms, male and female, as expressed by their ability when mated together to produce (i.e., bring to birth) individual offspring." According to this view, fertility depends upon and includes fecundity as well as a great number of other factors. As Pearl and Surface state: "Clearly it is fertility rather than fecundity which is measured in statistics of birth of mammals."

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The inbred strain of rats was composed of two series, A and B, both derived from the same ancestral stock. In every generation of each series the females that were used for breeding were paired twice with a brother from the same litter, thus producing the strictly 'inbred' litters that alone furnished the breeding stock in the following generation. These same females were then paired twice with an unrelated Albino male taken from the general stock colony. For convenience, litters with the latter parentage are here designated as 'half-inbred' litters.

The early generations of these inbred animals suffered severely from malnutrition, due to improper feeding. Nutritive conditions were improved after the fourth generation, and the animals quickly regained their normal size and fertility. At no stage of the investigation was any attempt made to influence the productiveness of the animals, other than by keeping them under environmental and nutritive conditions that were as uniform and as favorable as it was possible to make them.

A. Litter size

The normal fertility of any race can properly be estimated only from the total number of offspring produced by many females during the entire period of their reproductive activity. The fertility in the inbred strain of rats cannot be measured by this standard, unfortunately, since the plan of the experiment called for only four litters from each breeding female, and after this number was obtained the females were usually discarded. According to Crampe ('84), the Albino female has, on the average, only three or four litters. On this basis the litter data obtained for the inbred series shows the total productiveness of the greater proportion of the females that were used for breeding. Crampe's estimate for litter production is, I believe, too low, since the breeding history of a considerable number of stock Albinos, recently obtained, shows that the females had an average of 5.3 litters each. Records for the inbred series undoubtedly cover the most productive period in the life of the females, and if the fertility of the strain was impaired to any extent by inbreeding it is probable that all of the litters cast would have been smaller than normal.

The number and average size of the litters produced in each of the first twenty-five generations of the A series of inbreds are given in table 1.   Similar data for the litter production in the B series of inbreds are shown in table 2.

These tables are inserted chiefly for reference, but a comparison between corresponding data indicates clearly that the fertility of the animals in one inbred series was about the same as that in the other series.   The summary of the data for the two series shows that the 1752 litters in the A series contained an average of 7.5 young, while the 1656 litters in the B series had an average of 7.4 young.   This close agreement in the records for two such large groups of animals is doubtless due to the fact that all of the inbred rats were descended from the same ancestral stock and that individuals in corresponding generations of the two series were reared simultaneously under similar environmental conditions.

The data in table 1 and in table 2 have been combined in table 3, which thus shows the number and average size of the litters produced in the first twenty-five generations of the inbred strain. The data given comprise the records for 3408 litters containing 25,452 individuals.

To facilitate the discussion of the effects of inbreeding on fertility the data given in table 1 to table 3 were combined by generation groups.   There were relatively few individuals in the first six generations of inbreds and their data were united to form the first group, since the character of the experiment was changed at this point.   Data for subsequent generations were divided into five groups, each of which, with the exception of the last, comprised the records for four successive generations.   Such a division of the data was, of course, purely arbitrary, but it seemed the most satisfactory arrangement possible.   A group of four generations covers approximately the litter production for one year, and as the number and size of the litters vary considerably at different times of the year, this grouping assured a uniform distribution of the seasonal variations in litter size among all of the various groups.

Litter data for the A series of inbreds, arranged according to generation groups, are given in table 4.

TABLE 1

Showing the number and the average size of the litters produced in each of the first twenty-five generations of the A series of inbred rats

LITTER SERIES

GENER-ATION	First litters (inbred)			Second litters (inbred)			Third litters (half-inbred)			Fourth litters (half-inbred)			SUMMARY		
	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Total number of litters	Total number of individuals	Average number of young per litter
1	1	7	7.0	1	7	7.0	1	6	6.0	1	9	9.0	4	29	7.2
2	3¹	20	6.7	4	27	6.5	3	19	6.3	3	14	4.6	13	80	6.1
3	7	35	5.0	5	26	5.2	5	27	5.4	5	21	4.2	22	109	5.0
4	13	58	4.4	12	77	6.4	8	52	6.5	6	46	7.6	39	233	6.0
5	18	117	6.5	18	130	7.2	15	111	7.4	10	58	5.8	61	416	6.8
6	15	94	6.2	15	102	6.8	14	86	6.1	11	63	5.7	55	345	6.2
7	16	101	6.3	16	129	8.0	15	109	7.2	9	61	6.7	56	400	7.1
8	17	122	7.1	17	142	8.3	15	100	6.6	8	56	7.0	57	420	7.3
9	17	105	6.2	17	123	7.2	16	121	7.5	12	76	6.3	62	425	6.8
10	20	131	6.5	20	156	7.8	20	161	8.0	17	137	8.0	77	585	7.6
11	21	144	6.8	21	170	8.1	20	164	8.0	18	135	7.5	80	613	7.6
12	20	145	7.2	20	154	7.7	19	152	8.0	17	140	8.2	76	591	7.7
13	22	160	7.2	22	180	8.1	21	156	7.4	20	157	7.8	85	653	7.6
14	21	139	6.6	21	188	8.9	21	182	8.1	18	136	7.5	81	645	7.9
15	23	171	7.4	23	193	8.3	21	173	8.2	17	136	8.0	84	673	8.0
16	21	143	6.8	21	165	7.9	18	121	6.7	10	62	6.2	70	491	7.1
17	27	210	7.7	27	244	9.0	25	213	8.5	23	178	7.7	102	845	8.2
18	23	168	7.3	23	197	8.5	19	164	8.6	15	110	7.3	80	639	7.9
19	23	147	6.3	23	178	7.7	22	172	7.8	15	118	7.8	83	615	7.4
20	27	193	7.1	27	224	8.3	23	149	6.5	17	130	7.6	94	696	7.4
21	27	199	7.4	27	217	8.0	26	223	8.5	22	187	8.5	102	826	8.1
22	27	203	7.5	27	234	8.6	24	218	9.0	17	113	6.6	95	768	8.0
23	25	186	7.4	25	183	7.3	22	161	7.4	19	142	7.4	91	672	7.3
24	25	184	7.3	25	200	8.0	22	177	8.0	21	140	6.6	93	701	7.5
25	26	173	6.6	26	203	7.8	23	166	7.2	15	104	6.9	90	646	7.1
1-25	485	3,355	6.9	483	3,849	7.9	438	3,383	7.7	346	2,529	7.3	1,752	13,116	7.5

¹ One female destroyed her first litter.

TABLE 2

Showing the number and the average size of the litters produced in each of the first twenty-five generations of the B series of inbred rats

LITTER SERIES

GENER-ATION	First litters (inbred)			Second litters (inbred)			Third litters (half-inbred)			Fourth litters (half-inbred)			SUMMARY		
	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Total number of litters	Total number of individuals	Average number of young per litter
1	1	5	5.0	2	19	9.5	2	17	8.5	1	9	9.0	1	5	5.0
2	2	12	6.0	5	26	5.2	2	9	4.5				7	57	8.1
3	7	36	5.1	8	69	8.6	7	56	8.0	3	18	6.0	14	71	5.0
4	11	66	6.0	20	158	7.1	18	150	8.3	13	95	7.3	29	209	7.2
5	20	133	6.5	15	118	7.8	14	102	7.2	8	74	9.2	71	536	7.5
6	15	106	7.0	15	105	7.0	15	104	6.9	10	78	7.8	52	401	7.7
7	15	79	5.8	15	129	8.6	13	94	7.2	3	21	7.0	55	366	6.6
8	15	108	7.6	20	183	9.0	17	127	7.4	10	49	4.9	46	352	7.6
9	20	126	6.2	17	145	8.5	16	107	6.6	14	93	6.6	67	485	7.2
10	17	104	6.1	19	128	6.7	18	138	7.6	18	149	8.2	64	449	7.0
11	19	112	5.9	20	175	8.7	19	154	8.1	12	68	5.6	74	527	7.1
12	20	139	6.9	21	168	8.0	19	150	7.9	13	104	8.0	71	536	7.5
13	21	154	7.3	21	135	6.4	20	161	8.0	18	132	7.3	74	576	7.7
14	21	142	6.7	20	138	6.9	20	173	8.6	17	140	8.2	80	570	7.1
15	20	131	6.5	24	184	7.6	22	190	8.6	19	132	6.9	77	582	7.5
16	24	158	6.5	22	170	7.7	21	173	8.2	17	113	6.6	89	664	7.4
17	22	158	7.1	23	180	7.8	20	171	8.5	20	149	7.4	82	614	7.4
18	23	168	7.3	24	221	9.2	22	195	8.8	13	96	7.4	86	668	7.7
19	24	186	7.7	26	179	6.8	21	157	7.4	11	88	8.0	83	698	8.4
20	26	174	6.7	24	189	7.8	18	138	7.6	16	112	7.0	84	598	7.1
21	24	176	7.3	26	210	8.0	24	211	8.8	19	139	7.3	82	615	7.5
22	26	174	6.7	22	195	8.8	20	145	7.2	15	126	8.4	95	734	7.8
23	22	181	8.2	27	189	7.0	26	175	6.7	17	114	6.7	79	647	8.1
24	27	207	7.6	26	193	7.4	25	179	7.1	20	136	6.8	97	685	7.0
25	26	184	7.0										97	692	7.1
1-25	468	3,219	6.9	462	3,606	7.8	419	3,276	7.8	307	2,235	7.2	1,656	12,336	7.4

TABLE 3

Showing the number and average size of the litters produced in each of the first twenty-five generations of the two inbred series (A,B): a combination of the data in table 1 and in table 2

GENER-ATIONS	First litters (inbred)			Second litters (inbred)			Third litters (half-inbred)			Fourth litters (half-inbred)			SUMMARY		
	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Total number of litters	Total number of individuals	Average number of young per litter
1	2	18	6.0	1	7	7.0	1	6	6.0	1	9	9.0	5	34	6.8
2	5	32	6.4	6	46	7.6	5	36	7.2	4	23	5.7	20	137	6.8
3	14	71	5.1	10	52	5.2	7	36	5.1	5	21	4.2	36	180	5.0
4	24	124	5.1	20	146	7.3	15	108	7.2	9	64	7.1	68	442	6.5
5	38	250	6.6	38	288	7.6	33	261	7.9	23	153	6.6	132	952	7.2
6	30	200	6.6	30	220	7.3	28	188	6.7	19	137	7.2	107	745	6.9
7	31	180	5.8	31	234	7.5	30	213	7.1	19	139	7.3	111	766	6.9
8	32	230	7.2	32	271	8.5	28	194	6.9	11	77	7.0	103	772	7.5
9	37	231	6.2	37	306	8.3	33	248	7.5	22	125	5.7	129	910	7.0
10	37	235	6.3	37	301	8.1	36	268	7.4	31	230	7.4	141	1,034	7.3
11	40	256	6.4	40	298	7.4	38	302	8.0	36	284	7.9	154	1,140	7.4
12	40	284	7.1	40	329	8.2	38	306	8.1	29	208	7.2	147	1,127	7.6
13	43	314	7.3	43	348	8.0	40	306	7.6	33	261	7.9	159	1,229	7.7
14	42	281	6.7	42	323	7.7	41	343	8.4	36	268	7.5	161	1,215	7.5
15	43	302	7.0	43	331	7.7	41	346	8.4	34	276	8.1	161	1,255	7.8
16	45	301	6.7	45	349	7.7	40	311	7.6	29	194	6.0	159	1,155	7.2
17	49	368	7.5	49	414	8.4	46	386	8.4	40	291	7.2	184	1,459	7.9
18	46	336	7.3	46	377	8.2	39	335	8.5	35	259	7.4	166	1,307	7.8
19	47	333	7.1	47	399	8.5	44	367	8.3	28	214	7.6	166	1,313	7.9
20	53	367	6.9	53	403	7.6	44	306	7.0	28	218	7.7	178	1,294	7.2
21	51	375	7.3	51	406	8.0	44	361	8.2	38	299	7.9	184	1,441	7.8
22	53	377	7.0	53	444	8.3	48	429	8.9	36	252	7.0	190	1,502	7.9
23	47	367	7.8	47	378	8.0	42	306	7.3	34	268	7.9	170	1,319	7.7
24	52	391	7.5	52	380	7.5	48	352	7.3	38	254	6.7	190	1,386	7.3
25	52	357	6.8	52	396	7.6	48	345	7.2	35	240	6.9	187	1,338	7.2
1-25	953	6,574	6.9	945	7,455	7.9	857	6,659	7.8	653	4,764	7.3	3,408	25,452	7.5

TABLE 4

Showing for various generation groups the number and average size of the litters produced in the A series of inbred rats

| GENER-ATIONS | LITTER SERIES | | | | | | | | | | | | SUMMARY | | |
| | First litters (inbred) | | | Second litters (inbred) | | | Third litters (half-inbred) | | | Fourth litters (half-inbred) | | | | | |
	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Total Number of litters	Total Number of individuals	Average number of young per litter
1-6	57	331	5.8	55	369	6.7	46	301	6.5	36	211	5.9	194	1,212	6.2
7-10	70	459	6.5	70	550	7.8	66	491	7.4	46	330	7.1	252	1,830	7.2
11-14	84	588	7.0	84	692	8.2	81	654	8.0	73	568	7.8	322	2,502	7.7
15-18	94	692	7.3	94	799	8.5	83	671	8.0	65	486	7.5	336	2,648	7.8
19-22	104	742	7.1	104	853	8.2	95	762	8.0	71	548	7.7	374	2,905	7.7
23-25	76	543	7.1	76	586	7.7	67	504	7.5	55	386	7.0	274	2,019	7.4
1-25	485	3,355	6.9	483	3,849	7.9	438	3,383	7.7	346	2,529	7.3	1,752	13,116	7.5

An examination of table 4 shows that all of the litters produced in the first generation group of the A series were smaller, on the average, than corresponding litters in the later generation groups. The relatively low fertility of the animals in the early generations was not due to inbreeding, but to the fact that these individuals suffered from malnutrition. As soon as the nutritive conditions were improved there was at once an increase in the number and in the size of the litters produced, as the data for the fifth and for the sixth inbred generations show (table 1).

As indicated in the last column of table 4, the groups comprising the tenth to the twenty-fifth generations of the A series showed, as a whole, comparatively little variation in the average size of the litters. The maximum average size (7.8) came in the group including the fifteenth to the eighteenth generations. This maximum was, however, only 0.1 greater than the average litter size for the preceding and for the following group, and therefore it can have little, if any, significance.

Litter data for various generation groups in the B series of inbreds are shown in table 5.

As the average size of the litters produced in the first generation group was greater than that in the second group (table 5), it might appear that the fertility of the breeding females in the B series was not lessened by malnutrition. In the beginning of these experiments many more females of the B series than of the A series were completely sterile, but the females of the B series that did breed were the more productive. Malnutrition, in this instance, was a selective agent that helped to eliminate the tendency to sterility in the B series by preventing the breeding of any except the most fertile females.

In the B series the maximum average size of the litters was found in the group comprising the nineteenth to the twenty-second generations, but, as was the case in the A series, this maximum was not great enough to be considered significant.

Litter data given in table 4 and in table 5 have been combined in table 6.

The data for each of the two inbred series, as well as that given in table 6, shows that in all generation groups the first litter cast

TABLE 5

Showing for various generation groups the number and average size of the litters produced in the B series of inbred rats

GENER-ATIONS	LITTER SERIES												SUMMARY		
	First litters (inbred)			Second litters (inbred)			Third litters (half-inbred)			Fourth litters half-inbred					
	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Total number of litters	Total number of individuals	Average number of young per litter
1–6	56	358	6.3	50	390	7.8	43	334	7.7	25	196	7.8	174	1,278	7.3
7–10	67	417	6.2	67	562	8.4	61	432	7.0	37	241	6.5	232	1,652	7.1
11–14	81	547	6.7	81	606	7.4	76	603	7.9	61	453	7.4	299	2,209	7.4
15–18	89	615	6.9	89	672	7.5	83	707	8.5	73	534	7.3	334	2,528	7.5
19–22	100	710	7.1	100	799	8.0	85	701	8.2	59	435	7.3	344	2,645	7.6
23–25	75	572	7.6	75	577	7.7	71	499	8.4	52	376	7.2	273	2,024	7.4
1–25	468	3,219	6.9	462	3,606	7.8	419	3,276	7.8	307	2,235	7.2	1,656	12,336	7.4

TABLE 6

Showing for various generation groups the number and average size of the litters produced in the two inbred series (A,B): a combination of the data in table 4 and in table 5

LITTER SERIES

GENER-ATIONS	First litters (inbred)			Second litters (inbred)			Third litters (half-inbred)			Fourth litters (half-inbred)			SUMMARY		
	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Number of litters	Number of individuals	Average number of young per litter	Total Number of litters	Total Number of individuals	Average number of young per litter
1- 6	113	689	6.1	105	759	7.2	89	635	7.0	61	407	6.9	368	2,490	6.8
7-10	137	876	6.4	137	1,112	8.1	127	923	7.2	83	571	6.9	484	3,482	7.2
11-14	165	1,135	6.8	165	1,298	7.9	157	1,257	8.0	134	1,021	7.6	621	4,711	7.5
15-18	183	1,307	7.1	183	1,471	8.0	166	1,378	8.3	138	1,020	7.4	670	5,176	7.7
19-22	204	1,452	7.1	204	1,652	8.1	180	1,463	8.1	130	983	7.6	718	5,550	7.7
23-25	151	1,115	7.4	151	1,163	7.7	138	1,003	7.3	107	762	7.1	547	4,043	7.4
1-25	953	6,574	6.9	945	7,455	7.9	857	6,659	7.8	653	4,764	7.3	3,408	25,452	7.5

was the smallest in the litter series, as a rule; the second litter
was the largest; the third and fourth litters were intermediate in
size between the first and the second; a similar relation in the size
of litters has been found, also, in two groups of stock Albinos.
In both inbred series the first two litters cast in each generation
were the offspring of brother and sister matings; the third and
fourth litters were produced by the mating of an inbred female
with an unrelated stock male.   In the first of the stock series
noted (King and Stotsenburg, '15, table 7) all of the litters were
produced by the pairing of unrelated stock animals; in the second
stock series, for which data are given in table 7 of the present
paper, all of the litters obtained were the offspring of brother

TABLE 7

*Showing the average size of each of the first four litters produced by a series of stock
Albino females*

LITTER SERIES	NUMBER OF LITTERS	NUMBER OF INDIVIDUALS	AVERAGE NUMBER OF YOUNG PER LITTER
1	116	717	6.2
2	116	843	7.3
3	103	671	6.5
4	89	587	6.6
	424	2818	6.7

and sister matings.   Since in all three groups the average size
of the litters in the litter series varied in a similar way, it is evi-
dent the litter size does not depend at all on the relatedness or
the unrelatedness of the parents, but chiefly on the age of the
female.   Young females tend to be somewhat less prolific than
older ones, as Crampe ('83) noted.   The litters reach their
maximum size when the females are about five months old, but
the number of young does not decrease appreciably in the various
litters cast until the females have passed the height of their repro-
ductive power at about seven months of age (King, '16b).

As shown in the first paper of this series (King, '18), the rats
in the seventh to the ninth inbred generations were considerably
heavier, at any given age, than the individuals belonging to sub-
sequent generations.   The cause for this unusually vigorous

growth was attributed to a stimulation of the growth processes produced by adequate nutrition following a period of semi-starvation. During this period the productiveness of the females was increased considerably, since the average size of the litters in the group comprising the seventh to the tenth generations was 0.4 greater than the average for the previous generation group (table 6). The period of maximum fertility in the inbred series did not, however, coincide with the period of maximum growth in body weight, but came at a much later time (fifteenth to the twenty-second generations), when the litters contained 7.7 young, on the average. The fact that the average size of the litters in the last three generations of the inbred series was slightly lower than the maximum can be attributed to a change in diet made necessary by the economic conditions of the present time. This diet does not seem to be quite as favorable to growth and fertility as was the more varied diet used until the beginning of last year.

The graph in figure 1, showing the average size of the litters produced in the various generations of the inbred strain, was constructed from the data in the last column of table 3.

Starting at the point of 6.8, the graph in figure 1 drops at the third generation to 5.0, the lowest point in its course. From this point it rises slowly, and after the fifth generation tends to be a fairly horizontal line, since it never falls below 6.9 nor does it rise above 7.9. At more or less regular intervals the graph drops slightly below the normal level. The most pronounced depression is at the point of the third generation; a second drop comes at the ninth generation; other depressions of about the same depth are found at the point of the sixteenth, the twentieth, and the twenty-fourth generations. As the last three depressions in the graph occur at intervals of four generations, it is evident that they were not due to a chance variation in the data, but that they must express periodic changes in the reproductive cycle of the females that tended to reduce the number of young born. In whatever way this reduction was effected, whether by a lessening of fecundity or by limiting the number of embryos that were capable of normal development, the cause for it, I believe, lay in the seasonal changes in temperature which always have a marked effect on the

physical condition of the animals.   During the summer months rats suffer severely from excessive humidity and from high temperature, since their mechanism for heat regulation, under these conditions, is inadequate.   At this season their sexual activity is at its lowest point, and the litters that are produced tend to be relatively small.   Severe cold checks reproduction, but litters born under these conditions are usually of normal size and the
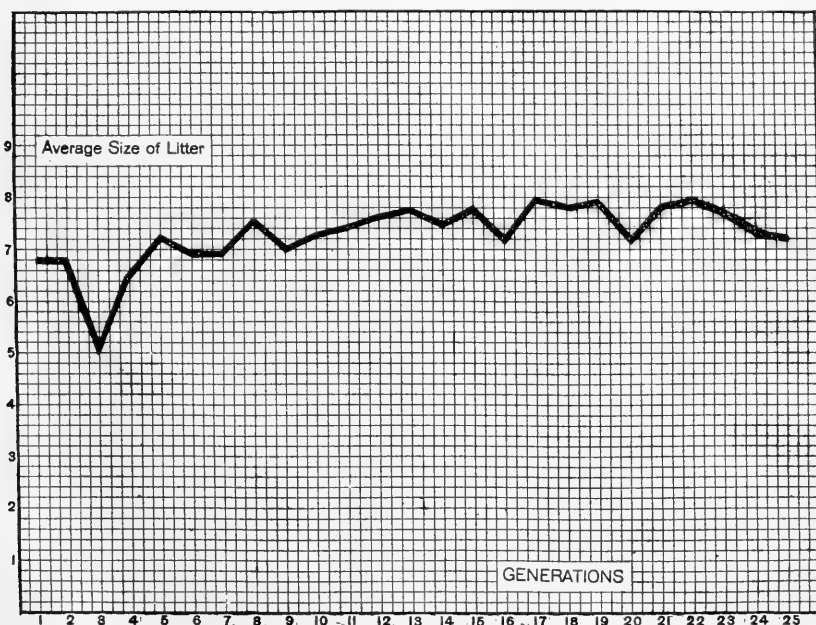
Fig. 1   Graph showing the average size of the litters produced in the various generations of the inbred strain (data in table 3).

individuals strong and vigorous.   At about every fourth generation the majority of litters produced in the inbred strain were born at the most unfavorable season of the year, the summer and early fall.   In this generation, as the records show, the litters were smaller, as a rule, than the litters in the preceding and in the following generations.   This decrease in size was sufficient to account for all of the depressions in the graph in figure 1, except the first one, which was doubtless due to the fact that the maxi-

mum effect of malnutrition in lowering the fertility of the females
was reached at the third generations.

Cyclic changes in productiveness were noted by Castle et al.
('06) in an inbred strain of Drosophila, in which, for three suc-
cessive years, there was a gradual rise in fertility followed by an
abrupt decline. These changes in productiveness were likewise
ascribed to the variations in temperature at different seasons of
the year.

The data given in table 1 to table 6 and the graph in figure 1
show clearly that, despite all theories to the contrary, it is possible
to maintain a high degree of fertility in a mammal for at least
twenty-five generations of the closest possible form of inbreeding,
by a careful selection of breeding stock and by keeping the ani-
mals under environmental conditions that are favorable for their
growth and reproduction.

While, in general, the size of the litter varies according to the
age of the mother, individual females differ greatly regarding the
number of offspring that they produce in any litter of the litter
series. Sisters from the same litter, mated to the same male,
will show marked variations in their fertility at the same age.
One female may never have a litter that contains more than five
young; the other may always throw litters in which there are
nine or more young. Some females, regardless of their age, tend
to cast the same number of offspring in every litter. One female
so noted had ten young in each of her four litters. Marked indi-
vidual differences in fertility are also found among female
guinea-pigs, according to Minot ('92).

The average number of young in a litter of albino rats is 6.3,
according to the data for 394 litters collected by Crampe ('84);
Cuénot ('99) found an average of 8.5 young in the 30 litters that
he examined. Records for 1089 litters of stock Albinos born in
the Wistar Institute animal colony during the years 1911 to 1914
give 7.0 as the average number of young per litter (King and
Stotsenburg, '15). When this last series of data was collected
it was not realized that litter size in the rat depends to such a
marked degree upon the age of the mother, and that in this species
the maximum fertility comes at a relatively early age, as it does

in the human race (Powys, '05) and also in poultry (Pearl, '17). Most of the litters recorded were cast by young females that had not reached the height of their reproductive power; such litters tend to be larger than those cast after this time (King, '16b). Data for litters cast by females of unknown age, however extensive they may be, cannot, therefore, properly be used to furnish the norm for litter size in the albino rat.

In order to obtain standards for litter size with which the data in the inbred strain might justly be compared, the complete breeding history of a considerable number of stock Albino females was recorded during the past three years. Data for the first four litters produced by 116 females belonging to this group are given in table 7. All of the stock rats from which these litters were obtained were reared under the same environmental conditions as the inbred strain.

In table 7, as has already been noted, the litters of the series bear the same size relation to each other as that found in the litter series of the inbred rats. The first litter was the smallest, averaging 6.2 young; the second litter, with an average of 7.3 members, was the largest of the series; while the third and fourth litters were somewhat smaller than the second. The entire series of 424 litters gave an average of 6.7 young per litter. This average is 0.3 less than that in the random collection of stock litters previously recorded (7.0), and 0.4 more than the norm as given by Crampe (6.3), so it is probably a fair standard for litter size in any similar series of Albino litters. There is no reason to believe that the stock females from which the litters recorded in table 7 were obtained were, as a group, inferior in reproductive power to other stock females, and presumably their fertility at any given age is fairly representative of that in the general run of stock Albinos.

Each litter of the stock series, shown in table 7, contained a smaller average number of young than the corresponding litter in either of the two inbred series when the data were arranged according to generation groups (tables 4 and 5), and, omitting the records for the first five generations where the fertility was lessened by malnutrition, there was not a single generation in either

of the inbred series where the average size of the first four litters was as low as that in the stock series (tables 1 and 2). In the inbred series as a whole, the average size of the litters was 0.8 greater than that in the stock series. Even if the previous finding of 7.0 be taken as the norm for litter size in the rat, the difference between the average size of the litters in the inbred strain and the norm chosen is 0.5. This difference is great enough to preclude the possibility that it was due to chance, and it cannot be attributed to the differential action of environment, since stock and inbred rats were constantly under the same environmental conditions. According to these findings, fertility in the inbred strain of Albinos, in as far as it may be judged by the size of the first four litters cast by a large number of females, was greater than the fertility in stock Albinos that were not inbred.

B. Frequencies of litter size

According to the several series of observations that have been recorded, there is a wide range in the size of the litters cast by Albino females. Kolazy ('71) reports litters containing from five to seventeen young, although Crampe ('84) states that he never found even fourteen young in a litter of albino rats. Litter size varied from four to twelve in the series of Albinos studied by Kirkham and Burr (15); while in the litters recorded by King and Stotsenburg ('15) the range in size was from two to fourteen.

Data for litter frequencies in the two series of inbred rats are shown in table 8.

TABLE 8

Showing the frequencies of litter size in the two series of inbred rats

	SIZE OF LITTER																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A	1	40	59	103	193	182	288	268	258	181	99	45	27	5	2	0	1
B	0	35	72	102	168	206	263	260	208	152	110	49	22	6	3	0	0
	1	75	131	205	361	388	551	528	466	333	209	94	49	11	5	0	1

In the A series of inbreds the range in litter size was from one to seventeen.   The litter of one was cast by a female of the nineteenth generation that was suffering from pneumonia and had to be killed three days after parturition.   This is undoubtedly a case where the physical condition of the female prevented the normal development of all of the embryos except one; the other embryos probably became atrophic and were absorbed.   The litter containing seventeen members occurred in the fifteenth generation.   All of the individuals were born alive, but they were all very small, weighing not more than three grams each: the average weight of the albino rat at birth is about four grams (King, '15b).

In the B series, as table 8 shows, the range of variation in litter size was not as great as that in the A series: no litters smaller than two or larger than fifteen were obtained.   In both series litters containing seven young were the most frequent, while those with eight young were only slightly less in number.

Figure 2 shows graphs for litter frequencies in the two series of inbreds that were constructed from the data given in table 8.

In figure 2 each graph rises quickly to the modal point at seven, falls slowly at first and then rapidly.   The drop in graph A at the point of 6 has apparently no significance, since there is no similar drop in the B graph.   Each graph is a simple frequency curve with one modal point, and is exactly the sort of graph that one would expect to obtain from the data for litter frequencies in a large series of animals belonging to a pure race.

C. Puberty

Under normal conditions puberty tends to appear at approximately the same age in the different individuals of a given race, but the time of its appearance is seemingly more dependent on the growth changes incident to age than it is on age itself.

In the albino rat both males and females attain sexual maturity when they are about two months of age (Donaldson, '15), but the environmental and nutritive factors that hasten or retard growth have considerable influence on the reproductive activity of the
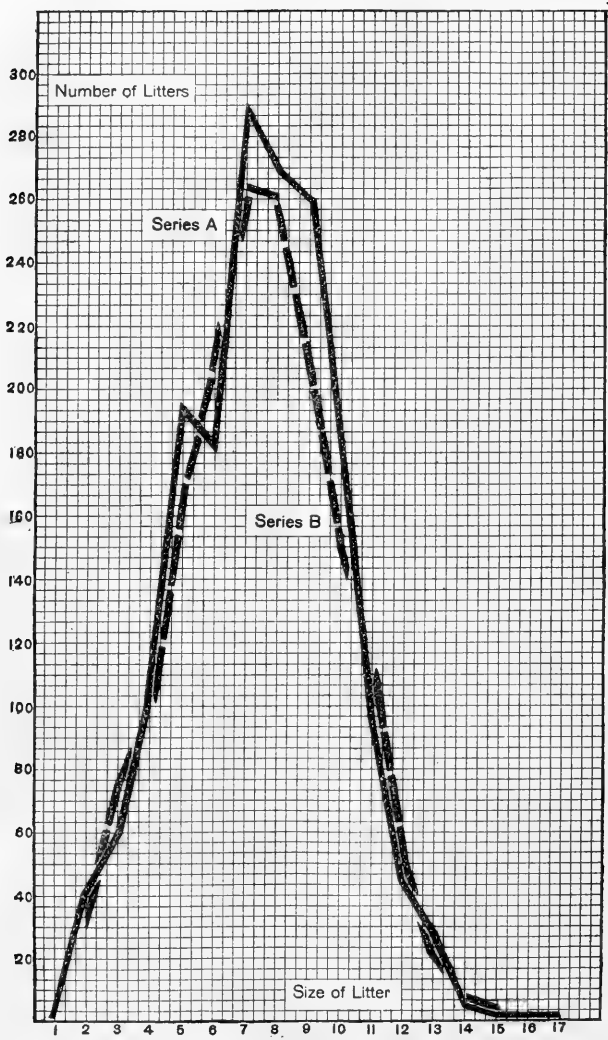
Fig. 2 Graphs showing the frequencies of litter size in the two series of inbred rats (data in table 8).

individuals.   If young rats are fed exclusively on a meat diet, puberty is considerably delayed (Watson, '06); the same effect is produced by underfeeding (Osborne, Mendel and Ferry, '17). According to my observations, the time of year in which the animals are born affects their subsequent growth and also the time of their maturing.   Rats born in the winter and early spring grow rapidly, and usually breed at about three months of age; those born in the summer and autumn grow more slowly and comparatively few of the females cast litters before they are four months old, many not breeding until spring, which is the season of the most pronounced sexual activity for the rat.   Convincing evidence that age alone does not determine the beginning or the end of the reproductive life of the rat is given by Osborne and Mendel ('15, '17), who found that Albino females, stunted at an early age by underfeeding, were completely sterile until they were properly nourished, when they grew rapidly, attained a normal size, and were able to breed long after the age at which the menopause usually appears.

According to Darwin ('75) and others, favorable environment tends to delay sexual maturity, though not necessarily to decrease fertility.   Since these inbred rats were reared, for the most part, under environmental conditions that seemed well adapted to their needs, and since they lacked the stimulus to reproductive vigor which is said to come from outcrossing, it might be expected that they would tend to mature much later than stock Albinos which were not inbred.

Table 9 shows the approximate age at which the breeding females belonging to various generation groups of the two inbred series cast their first litter.

The records for the first generation group, given in table 9, confirm Osborne and Mendel's findings that underfeeding tends to retard sexual maturity, since they show that about one-half of the breeding females in this group did not cast their first litter until they were four months old.   In subsequent generations, when the animals were adequately nourished, they began breeding at a much earlier age.   Under the conditions of this experiment, inbreeding seemingly hastened the onset of puberty, for

in both series, as the inbreeding advanced, there was a marked tendency for relatively more of the females to breed at the earliest possible age. About 30 per cent of the breeding females in the eighteenth to the twenty-fourth generation group of each series threw their first litter at or before the age of ninety days; only a small proportion of them failed to breed before reaching the age of four months.

As a whole, the females of the A series of inbreds tended to mature slightly earlier than the females of the B series, but the

TABLE 9

Showing the approximate age at which breeding females in various generation groups of the two inbred series (A and B) cast their first litters

GENERATION GROUPS TO WHICH BREEDING MALES BELONGED	SERIES A				SERIES B				SUMMARY (A,B)			
	Total number of breeding females	Per cent females breeding before 90 days of age	Per cent of females breeding between 90 and 120 days of age	Per cent females breeding after 120 days of age	Total number of breeding females	Per cent females breeding before 90 days of age	Per cent females breeding between 90 and 120 days of age	Per cent females breeding after 120 days of age	Total number of breeding females	Per cent females breeding before 90 days of age	Per cent females breeding between 90 and 120 days of age	Per cent females breeding after 120 days of age
1– 5	58	12.0	36.2	51.8	56	14.3	41.1	44.6	114	13.1	38.7	48.2
6– 9	70	21.4	68.6	10.0	67	7.4	74.7	17.9	137	14.6	71.6	13.8
10–13	84	25.0	71.4	3.6	81	13.6	74.1	12.3	165	19.3	72.8	7.9
14–17	94	28.7	62.8	8.5	89	25.8	62.9	11.3	183	27.3	62.9	9.8
18–21	104	36.5	62.6	0.9	100	31.0	64.0	5.0	204	33.8	63.3	2.9
22–24	76	31.5	60.6	7.9	75	20.0	69.4	10.6	151	25.8	65.0	9.2
1–24	486	27.1	61.6	11.3	468	19.8	65.3	14.9	954	23.5	63.4	13.1

difference between the two series was not great, and corresponding records were in nearly as close agreement as were those for litter size.

The youngest breeding female in the inbred strain was a member of the A series of inbreds, and she was eighty days old when she cast her first litter of five young. As the gestation period in the albino rat is about twenty-two days (Donaldson, '15), this female must have conceived when she was two months old. Kirkham and Burr ('15) state that one of their Albino females gave birth to a litter when she was only seventy-seven

days old; while Lantz ('10) reports a case in which an albino rat was said to have produced a litter at the age of fifty-six days. This last case is certainly a remarkable one, and its parallel has not been found among the 50,000 rats bred in our colony.

The last section of table 9 shows that, after the tenth generation, there was no marked change in the proportion of females that bred at three and at four months of age, respectively. Nearly 24 per cent of the total number of females used for breeding cast their first litter by the time they were three months old; over 60 per cent of them bred for the first time when they were between ninety and one hundred and twenty days of age; while about 13 per cent did not breed until after they were four months old.   The latter group was made up, for the most part, of females that were born in the summer or autumn.

Although Düsing ('84) states that inbred animals tend to mature very early, I do not think that inbreeding alone was responsible for the fact that relatively more of the females in the later than in the earlier generations of these inbred rats bred at three months of age.   In these experiments, when two or more females of a litter were reared as possible breeding stock, the first female that became pregnant was the one taken to continue the line, provided she fulfilled all requirements as to size and vigor. Thus the manner in which breeding females were selected preserved those individuals that tended to breed at an early age, and this tendency to early maturity, if heritable, must have been retained in the stock and intensified to some extent through continued brother and sister matings.   Inbreeding, aided by selection, would thus seem to be the factor involved in producing a strain of rats in which the females attained sexual maturity at a relatively early age.

D. Sterility

Sterility occurs normally in the Albino, as in other strains of rats, and therefore it might be expected to appear at times in any strain, regardless of whether the animals were inbred or outbred.   Crampe ('84) states that of 221 Albino females which he selected for breeding forty-six, or 20.8 per cent, were sterile.

Out of 124 stock Albino females reared in our own colony during the past three years and intended for breeding purposes thirty-two, or 28.8 per cent, were completely sterile, while about 10 per cent of those that did breed cast only one or two litters. Unfortunately, no records have been kept that give information regarding the exact proportion of sterile females in the first six generations of the inbred series. The number was relatively very large, and must have included at least one-half of the total number of females that lived to be six months old. Sterility in these females was, for the most part, the result of poor nutrition, and it disappeared as soon as the nutritive conditions were improved. Loeb ('17) has shown that in the guinea-pig "under-feeding prevents maturation of the follicles and thus causes sterility which lasts as long as the effect of the underfeeding is present in the ovary." In the guinea-pig, as in the rat, adequate nutrition reëstablishes normal conditions in the ovary and sterility almost entirely disappears.

In Drosophila, according to Castle et al. ('06), low productive-ness (sterility) is directly transmitted by inheritance and is amenable in selection. In the rat, sterility seems to depend not entirely on genetic factors, but to a marked extent upon conditions, such as malnutrition and disease, that act unfavorably upon reproduction. In the present experiments, by selecting for breeding only the most vigorous individuals (which it seems were also the most fertile), sterility in as far as it may depend on genetic factors would seem to have been practically eliminated from the strain, and it has not reappeared even after twenty-eight generations of brother and sister matings.

Of the 954 inbred females that were used for breeding during the course of these experiments, 653, or 68.5 per cent, cast four litters each, and many of them, kept for body-weight records, produced several other litters which were not recorded. Of the females that did not cast the required four litters, the great majority died from pneumonia, or were killed because they showed unmistakable evidence of illness. A few of the females stopped breeding after producing one or two litters, although they were apparently in good physical condition and were paired for several

months with males that were known to be fertile.   A postmortem examination of the reproductive organs from several of these semi-sterile females showed, in every instance, an inflamed condition of the ovaries or of the uterus which would render reproduction impossible.   Barrenness in these cases was doubtless due to disease and not to any inherent tendency to sterility.   A similar diseased condition of the reproductive organs has been found to be responsible for the partial sterility of stock Albinos.

2. THE CONSTITUTIONAL VIGOR OF INBRED RATS

The best criterion by which to gauge the so-called 'constitutional vigor' of any animal is undoubtedly its power of reproduction, since that is of the utmost importance for the continuation of the race.   There are, however, other important tests for vigor that can be applied, such as the rate and extent of growth, agility, mental alertness, resistance to disease, and ability to live to an advanced age.   According to Darwin ('78), "the effects of close interbreeding in animals, judging from plants, would be deterioration in general vigor, including fertility, with no necessary loss of excellence of form."   This would seem to indicate that, whatever tests were applied, closely inbred animals and plants would show marked inferiority when compared with individuals of the same species that were not inbred.   That such a sweeping generalization is not justified is shown by the results of a number of recent inbreeding experiments: the work of Shamel ('05) on tobacco, of Stout ('16) on chicory, and of Hayes and Jones ('17) on tomatoes give no indication that self-fertilization in these plants causes a loss either of vegetative or of reproductive vigor; Gentry's ('05) experiments on swine, and those of Castle et al. ('06) and of Moenkhaus ('11) on Drosophila show that any loss of vigor that might come from inbreeding can be entirely overcome by the proper selection of breeding stock.

The present series of experiments on the rat are the first recorded for any mammal in which brother and sister matings were made continuously for twenty-five successive generations. In the inbreeding experiments with rodents made by Crampe, by Ritzema-Bos, and by von Guaita, matings were made between

animals related in various degrees, and they were made as often between parent and offspring as between sibs. Ritzema-Bos states: "Bemerkenswert ist namentlich das Result, dass die Paarung zwischen Geschwistern viel schlechtere Erfolge lieferte als die Paarung zwischen Mutter und Sohn, resp. Vater und Tochter." Presumably, therefore, my inbred strain, in which all breeding females came from litters produced by the matings of sibs only, would show an even greater evidence of deterioration in vigor than did the rats inbred by Crampe and by Ritzema-Bos.

Data already given show that these inbred rats were much more fertile than stock rats reared under the same environmental conditions, so it is evident that their reproductive vigor was not impaired. In their ability to withstand disease inbred rats compared favorably with stock rats. The rat scourge, pneumonia, was quite as prevalent among stock animals as among the inbreds and took its toll of lives as frequently and as quickly in one strain as in the other. Parasitic infection was as common in the stock colony as in the inbred, and severe changes in temperature were followed by just as many deaths among stock animals as occurred in the inbred strain. The rat's power of resistance to disease and to unfavorable environmental conditions did not appear to be lessened by inbreeding under the conditions of these experiments.

Records for the growth in body weight of a considerable number of rats belonging in various generations of the two inbred series show the approximate age at which death occurred in all individuals that did not live to the end of the weighing period, which came when the rats were fifteen months old. As similar records were recently obtained for a series of stock animals, it is possible to compare the relative length of life in the two strains and thus to determine whether inbreeding tends to shorten the life of the individuals, as it might be expected to do if it impaired the general vigor of the animals to any extent.

Table 10 shows the mortality at different ages in such of the A series of inbreds as were used for the determination of the effects of inbreeding on the growth in body weight, given in the first paper of this series. For convenience the data were arranged in generation groups: the last group includes the findings through

the twenty-third generation only, as the weight records for animals belonging in the twenty-fourth and in the twenty-fifth generations are not yet completed. As all of the animals reached the age of three months, the first mortality record given is that for animals at six months of age.

On examining the mortality data for the males, as given in table 10, it is found that comparatively few of the animals in any generation group died before the age of six months, and that over 50 per cent of them lived to be more than one year old. A comparison of the corresponding records for the various generation groups shows unmistakably that the animals belonging to

TABLE 10

Showing the mortality at different ages in a group of 236 males and 179 females belonging in the seventh to the twenty-third generations of the A series of inbred rats

GENERATION GROUPS	NUMBER OF MALES	PER CENT MALES LIVING AT VARIOUS AGES				NUMBER OF FEMALES	PER CENT FEMALES LIVING AT VARIOUS AGES			
		6 mos.	9 mos.	12 mos.	15 mos.		6 mos.	9 mos.	12 mos.	15 mos.
7–10	35	91.4	71.4	54.3	45.7	28	92.8	67.8	25.0	10.7
11–14	52	90.3	71.1	57.7	38.4	37	97.3	83.8	56.7	35.1
15–18	60	100.0	75.0	53.3	26.6	47	97.8	72.3	59.5	29.8
19–23	89	98.8	88.7	73.0	46.0	67	98.5	82.1	67.1	46.2
7–23	236	96.2	78.8	61.9	39.4	179	97.2	77.6	56.4	34.1

the later generations tended to be longer lived than did those in the earlier generations.

The mortality data for the females of the A series are much like those for the males, the most noticeable difference being found in the records for the first generation group where only 10 per cent of the females lived to be fifteen months of age. Taking the animals of the A series as a whole, about 4 per cent of them died before they reached the age of six months; 20 per cent did not live to the age of nine months; 50 per cent were dead at the end of one year, and only about 35 per cent lived to be fifteen months old.

Mortality data for individuals belonging to various generation groups of the B series are shown in table 11.

In the earlier generations of the B series the mortality in both males and females was considerably greater than that in the animals belonging to the A series: only 5 per cent of the males lived to be fifteen months old, while not a single female reached this age. For the later generation groups the data for the B series were very similar to those for the A series. As a whole, however, the animals in the A series lived longer than did those in the B series.

The data given in table 10 and in table 11 have been combined in table 12. This table shows also mortality data for 377 stock albino rats reared in The Wistar Institute animal colony during the past three years. Included in the latter series are the

TABLE 11

Showing the mortality at different ages in a group of 151 males and 231 females belonging in the seventh to the twenty-third generations of the B series of inbred rats

GENER-ATION GROUPS	NUM-BER OF MALES	PER CENT MALES LIVING AT VARIOUS AGES				NUMBER OF FEMALES	PER CENT FEMALES LIVING AT VARIOUS AGES			
		6 mos.	9 mos.	12 mos.	15 mos.		6 mos.	9 mos.	12 mos.	15 mos.
7–10	18	94.4	50.0	38.8	5.5	34	79.4	23.5	17.6	
11–14	30	86.6	70.0	26.6	6.6	43	90.7	65.1	34.9	16.2
15–18	43	100.0	69.8	51.1	27.9	64	96.9	76.5	54.6	26.5
19–23	60	100.0	93.3	76.6	56.6	90	100.0	86.6	77.7	53.3
7–23	151	97.2	76.8	54.9	32.4	231	94.3	70.6	54.5	31.1

records, elsewhere published (King, '15), for fifty males and for fifty females of selected stock that were reared as controls for the inbred strain.

The mortality data for the inbred rats, given in table 12, show that close inbreeding did not tend to shorten, but to lengthen the span of life in both males and females: 50 per cent of the animals belonging to the last group lived to be fifteen months of age, while in none of the other groups did even 30 per cent of the individuals attain this age. It is probable that the relatively high death rate in the animals of the earlier generations was due to the fact that the rats had not regained the vigor that was so greatly impaired in their ancestors by malnutrition.

Donaldson ('06) has assumed that the span of life in man is thirty times that of the rat, and therefore that a rat of three years corresponds to a man of ninety years.   Considering the relatively small proportion of men that live to be nonagenarians, one would not expect to find many rats in any colony living to three years of age, yet under the equitable climate of California, Slonaker ('12) succeeded in keeping two of a series of sixteen albino rats beyond this age, and one of them lived for forty-five months, or the equivalent of one hundred and twelve years of human life.   At various times during the past five years a number

TABLE 12

Showing the mortality at different ages in a group of 387 males and 410 females belonging in the seventh to the twenty-third generations of the two inbred series (a combination of the data in table 10 and in table 11).   Data are also shown for the mortality in a series of stock albino rats comprising 199 males and 178 females

GENERATION GROUPS	NUM-BER OF GROUPS	PER CENT MALES LIVING AT VARIOUS AGES				NUM-BER OF FE-MALES	PER CENT FEMALES LIVING AT VARIOUS AGES			
		6 mos.	9 mos.	12 mos.	15 mos.		6 mos.	9 mos.	12 mos.	15 mos.
7–10	53	92.4	64.1	49.0	32.0	62	85.4	43.5	20.9	4.8
11–14	82	89.0	70.7	47.1	26.8	80	93.7	73.7	45.0	25.0
15–18	103	100.0	72.8	52.4	27.1	111	97.3	74.8	56.7	27.9
19–23	149	99.3	90.6	74.5	50.3	157	99.3	84.9	73.3	50.3
7–23	387	96.3	78.0	59.2	36.7	410	95.6	73.6	55.3	32.0
Stock series..	199	98.9	85.9	63.8	28.1	178	95.5	87.6	70.2	37.6

of inbred and of stock Albinos were kept in our colony in good physical condition until they were about two years old.   We have never attempted to keep any rats beyond this age.

Osborne, Mendel and Ferry ('17) state that out of ninety-one albino rats kept under ordinary laboratory conditions during their entire lifetime, "17 (19 per cent) died under one year of age; 48 (53 per cent) died between one and two years of age; and 26 (29 per cent) lived more than two years, the oldest one reaching an age of nearly 34 months.   From these figures it is evident that less than a third of the rats in our colony may be expected to live to be more than two years old."   In another paper these

authors ('15) state: "Fully half of our stock rats have died before the age of 600 days." Unfortunately, the mortality data given by Osborne, et al. are not in a form which makes it possible to compare them directly with the records for these inbred rats. It would seem, however, from the results as given, that their animals tended to live longer than the rats in my inbred strain. The mortality data for the series of stock Albinos, given in table 12, can be directly compared with those for the inbred rats given in the same table, since both series of animals were reared under similar environmental conditions and the records were taken at the same age intervals. Relatively more of the stock than of the inbred males were living at six, nine, and twelve months of age, but only 28 per cent of the stock males lived to be fifteen months old, while 37 per cent of the inbred males attained this age. The records for the female groups show that relatively as many inbred as stock females lived to the age of six months, but that more of the stock than of the inbreds were living at nine, twelve, and fifteen months of age. Taken as a whole, therefore, longevity in the inbred strain seemed to be somewhat less than that in the stock controls.

Some of the inbreeding data for animals which Darwin ('75) collected were so at variance with his own results on plants that he was forced to admit that; "manifest evil does not usually follow from pairing the nearest relations for two, three, or even four generations." In a long-continued series of inbreeding experiments, therefore, the deleterious effects of inbreeding would supposedly be more accentuated in the later than in the earlier generations. A comparison between the mortality records for stock animals and those for the inbred group comprising the animals in the nineteenth to the twenty-third generation should show the effects of inbreeding on longevity much better than the comparison between the groups as previously made. Such a procedure is the more justifiable, perhaps, because these two groups of animals were reared in the colony simultaneously. While in the two male groups only about 1 per cent of the animals failed to reach the age of six months, relatively more of the inbred than of the stock males were living at all other age periods noted:

the final records for the two groups show a difference of 22.2 per cent in favor of the inbred animals. In the female groups the span of life in the inbreds also tended to be longer than that in the controls, but the difference was not quite as marked as in the case of the males: the final records show a difference of only 12.7 per cent.

It appears, from the above comparison of data for stock and inbred rats, that continued inbreeding, under favorable environmental conditions and with the aid of selection, cannot only lessen the tendency to early death caused by malnutrition, but that it can extend the average span of life in the rat considerably beyond that found in the stock controls. Constitutional vigor, as judged by the longevity of the individuals, is therefore not invariably lessened by continued inbreeding.

In table 10 and in table 11 it will be noted that the mortality data for the first generation group indicate that the span of life in the females, particularly in the B series, was much shorter than that in the males. The reason for this 'selective mortality' is not clear, although it may be that the females were not able to throw off the effects of malnutrition quite as readily as were the males. In both inbred series, after the tenth generation, the mortality in the females at any age period was practically the same as that in the corresponding group of males. Data given in table 12 show that stock females tended to live longer than stock males: a reversed relation seemed to hold for the inbred rats. Taking the inbred colony as a whole, I am inclined to the opinion that the females, as a rule, tend to live longer than do the males. More males than females usually die as the result of a sudden, sharp change in temperature, and the impression one gets from working daily with the animals is that the males are far more susceptible to pneumonia than are the females, and that they are sooner attacked by various parasitic pests, such as lice and ear-mites. White ('14) states that in India the bubonic plague is a more fatal disease to male than to female rats, thus indicating that the female is stronger, constitutionally, than the male. These results are in accord with the findings for the human race: census reports and various statistical tables that have been com-

piled show, as does the investigation of Pearson et al. ('03), that the duration of life in women is longer than it is in men and that women are the less susceptible to disease at all ages.

The various physical defects, so prevalent among Crampe's ('83) inbred rats, were all found among my inbred rats at the beginning of these experiments, but they were due to malnutrition, not to inbreeding, since they entirely disappeared when the animals received proper food. Among the thousands of inbred animals that were reared during the past five years some few, not to exceed a dozen in all, lacked one or both eyeballs. This defect has also appeared, at times, in stock animals. On the average, one in every 10,000 rats born in the stock colony is tailless. This abnormality, as Conrow ('15, '17) has shown, involves the skeletal structure in the entire pelvic region. The inbred colony has contained only one tailless individual as yet. Unfortunately, this rat was destroyed by the mother soon after birth, so it was not carefully examined. Neither of these defects appears to be heritable, and neither can be due to inbreeding, since each has appeared also in a stock that is outbred. No other abnormalities of any kind have appeared in the animals of the inbred strain up to the present time when the individuals of the twenty-eighth generation are approaching maturity. The findings in this series of experiments, therefore, do not give support to Ritzema-Bos' contention that inbreeding tends to cause "eine grössere Prädisposition für Krankheiten und das Entstehen von Missbildungen." When a considerable number of animals belonging to any series exhibits various kinds of malformations, it is safe to assume that either environmental and nutritive conditions are unfavorable to normal development, as in the early part of the present series of experiments, or that there is an inherent weakness in the stock used that is brought out and accentuated by random inbreeding, as seemed to be the case with Crampe's rats.

No data are available for a direct comparison between stock and inbred rats as regards their relative activity at different ages, but several series of experiments have been made in different psychological laboratories in which the behavior of rats from this inbred strain was compared with that of stock controls.

In the inbred rats of the earlier generations the brain and spinal cord were decidedly below the normal weight of these organs in stock animals of like age and body weight. "From the fourth to the tenth generation the relative brain weight remained, on the average, constant at six and one-half per cent less than that of the normal control rats" (Basset, '14). The habit formation in a number of rats that belonged in the sixth and in the seventh inbred generations was tested at Johns Hopkins University by Basset ('14), who found that these animals were inferior to stock rats in their ability to form habits, and that they show less retention of a habit, and were longer in relearning it, than were the controls.

Inbred rats belonging in the twelfth and in the fourteenth generations were sent to Harvard University where Mrs. Yerkes ('16) studied their behavior and compared it with that of stock albino rats obtained from The Wistar Institute colony and from a different source of supply. The general conclusion reached by Mrs. Yerkes was that "inbred rats learned a trifle more slowly than the stock rats, both in the maze and in the discrimination experiments, but that they carried discrimination of lightness and darkness further, and showed the most pronounced difference only in their greater timidity and instability of behavior."

Temperamental differences between stock Albinos and inbreds of the fourteenth and the fifteenth generations were investigated at Harvard by Utsurikawa ('17). The results obtained showed that inbred rats were less active and more savage than the outbred rats, and that they responded more quickly and in greater amount to momentary auditory stimulation than did outbred rats. The two strains were found to differ also in "restlessness or continuity of response." Inbred rats showed the greatest restlessness" in case of momentary and repeated auditory stimulation and less in case of continued stimulation, whereas for the outbred animals the reverse is true." These temperamental differences between inbred and stock rats would seem to indicate that inbred rats are more 'high strung' nervously than are outbred rats. Nervousness is a trait manifested by many thoroughbred animals, and it is particularly characteristic of the racehorse.

The nervousness of the horse is undoubtedly the result of continued selection, since breeders consider that an animal must have this trait highly developed if it is to be a success on the track. If nervousness is a trait that is transmitted by inheritance and amenable to selection it is probably also a trait that would tend to be intensified by close inbreeding, and therefore it might be expected that rats closely inbred for many generations would be somewhat more nervous than outbred stock controls, as Utsurikawa found to be the case.

When the last two series of investigations were completed the animals used were sent to The Wistar Institute where they were killed and carefully examined by Dr. Hatai. It was found, as Mrs. Yerkes states, that the inbred rats had a somewhat greater body length and body weight than the stock rats, and that they showed a brain weight in relation to body length and body weight that was only from 0.002 per cent to 0.006 per cent less than that of stock rats. Since the inbred rats of the sixth and of the seventh generations had a brain weight about six and one-half per cent less than the normal (Basset, '14), it would appear, from Mrs. Yerkes' findings, that somewhere between the seventh and the twelfth generations the animals entirely recovered from the effects of malnutrition and became normal again with respect to the relative weight of the central nervous system. They have remained normal in this regard up to the present time, as autopsies made at various periods on animals of the later generations have shown.

With the return of the central nervous system to its normal weight relations, the inbred rats must have regained much of their lost mental vigor, since in behavior tests animals of the fourteenth generation were found to be inferior to stock animals only in that they were slower and less active. The lesser activity of the inbred rats Mrs. Yerkes ascribes to "a greater timidity and a greater susceptibility to environmental conditions." Savageness, wildness, and timidity are heritable behavior complexes, according to R. Yerkes ('13), and since no attempt was made in the course of these experiments to eliminate these traits by selection, it is not surprising that they were manifested in a somewhat intensified form after many generations of close inbreeding.

3. DISCUSSION

Wherever inbreeding has been practiced it has usually been accused of producing anything and everything undesirable that has appeared in the offspring. The following quotation from Mitchell ('65) is quite typical of the belief that prevailed among zoologists, as well as among the laity, until the past decade, regarding the effects of consanguineous marriages:

> Consanguinity in parentage tends to injure the offspring. This injury assumes various forms. It may show itself in diminished viability at birth; in feeble constitutions, exposing them to increased risks from the invasion of strumous disease in after life; in bodily defects and malformations; in deprivation or impairment of the senses, especially those of hearing and sight; and, more frequently than in any other way, in errors and disturbances of the nervous system, as in epilepsy, chorea, paralysis, imbecility, idiocy, and moral and intellectual insanity. Sterility or impaired reproductiveness is another result of consanguinity in marriage, but not one of such frequent occurrance as has been thought.

Stock breeders, also, have been imbued with the idea that inbreeding is always inimical to constitutional vigor and that it leads to sterility. For these reasons most of them have opposed the mating of animals related even in a remote degree. During the past few years it has been shown by a number of carefully controlled experiments that inbreeding does not necessarily produce the evil effects that have been attributed to it, and that the results obtained in any inbreeding experiment depend, primarily, on the soundness of the stock that is inbred; secondarily, on the selection of animals for breeding purposes, and, finally, on the environmental conditions under which the animals live. Haphazard inbreeding of inferior stock under unfavorable environmental conditions has produced many of the failures for which inbreeding alone has been held responsible.

Since the experiments of Crampe ('83), of Ritzema-Bos ('93, '94), and of von Guiata ('98, '00) have furnished the classic examples of the dire effects of inbreeding on rodents, it may be well to examine these experiments in some detail to see whether the unfavorable results obtained cannot be traced to some cause other than inbreeding per se.

Crampe's inbreeding experiments were begun, in 1873, with an Albino female and a white and gray male. From the mating of these rats he obtained the litter of five young which formed the basis of his breeding stock. These animals were inbred, in various degree of relationship, for seventeen successive generations. Crampe states that many of the animals were sterile and that others lost their reproductive instincts at the end of the first year. Various kinds of malformations appeared; the animals were seemingly too weak to resist disease of any kind, and they died at a relatively early age. The weakness of these rats and their susceptibility to disease, as well as the high degree of sterility among them, all point to the probability, as Ritzema-Bos suggests, that Crampe started his experiments with animals taken from a defective stock. Since results similar to Crampe's were obtained in the early part of my own experiments, I am inclined to the opinion that inadequate nourishment was a factor that was responsible, in great part, for his failure to maintain the stock in good physical condition.

Ritzema-Bos started his investigation in 1886 with a litter of twelve rats that was obtained from the mating of an Albino female and a wild Norway male. These rats were inbred, in various ways, for six years, during which time, Ritzema-Bos states, "about thirty generations were obtained." There is evidently some inaccuracy in this latter statement. The female albino rat does not cast her first litter until she is about three months old; wild rats do not breed, as a rule, before they are four or five months old. Assuming that all of the females used in Ritzema-Bos' experiments bred at the earliest possible age, i.e., three months, only four generations could possibly be produced in a year: this would give a maximum of twenty-four generations at the end of six years. In my own experiments an average of about three and one-half generations a year were obtained.

Ritzema-Bos gives data showing the average size of the litters and the number of infertile matings during the various years in which the work was in progress. These data have been reproduced in table 13.

During the first three years, as table 13 shows, there was little diminution in the average size of the litters produced.   In the three following years, however, litter size decreased considerably, and at the end of the investigation the litters averaged less than one-half the size of those obtained in the beginning.   These results certainly justify Ritzema-Bos' conclusion that: "Die fortgesetze Zucht in engster Verwandtschaft vermindert das Fortpflanzungsvermögen, kann sogar schliesslich vollkommene Unfruchtbarkeit verursachen."   Lloyd ('12) has suggested that the deterioration in Ritzema-Bos' stock might have been due to overcrowding, since many varieties of rats will not breed in close confinement.

TABLE 13

Showing Ritzema-Bos' data for the average size of the litters and for infertile matings in a series of inbred rats

YEAR	AVERAGE NUMBER OF YOUNG PER LITTER	PER CENT INFERTILE MATINGS
1887	7.5	0.00
1888	7.1	2.63
1889	7.1	5.55
1890	6.5	17.39
1891	4.2	50.00
1892	3.2	41.18

Von Guaita obtained a number of white mice from a strain that had been inbred by August Weismann for twenty-nine generations.   How these mice were inbred I do not know, since I have not been able to find any account of the details of this experiment. Von Guaita crossed these white mice with Japanese waltzing mice, and then inbred their descendants for five generations. The data regarding the average size of the litters obtained in these two sets of investigations are shown in table 14.

Weismann's data, given in table 14, show that the average size of the litters decreased directly as the inbreeding advanced, and so appear to indicate that inbreeding lessened the fertility of the mice.   In this experiment there seems to have been a very great difference in the number of litters that were produced in the various generations.   In the first two generations there was an aver-

age of about twenty-two litters to the generation: in the last nine generations the average was only about three litters to a generation. Such a small number of litters as that produced in the later generations of this series does not afford an opportunity for a careful selection of breeding stock, neither does it furnish sufficient data to make the results of statistical value.

In the successive generations of mice bred by von Guaita there was, to quote Davenport ('00): "a reduction in fertility of about 30 per cent, and this is probably due to close inbreeding." In order to make this deduction from von Guaita's data, however, it

TABLE 14

Showing the number and average size of the litters in twenty-nine generations of white mice inbred by August Weismann, and in seven generations of hybrid mice inbred by von Guaita

	GENERATIONS	NUMBER OF LITTERS	AVERAGE NUMBER OF YOUNG PER LITTER
Weismann's data for white mice........	1–10	219	6.1
	11–20	62	5.6
	21–29	29	4.2
Von Guaita's data for hybrid mice.....	1	7	4.4
	2	15	3.0
	3	25	3.8
	4	31	4.3
	5	30	3.2
	6	11	2.3

is necessary to combine the records for three generations, as Davenport did. If the records for the various generations are considered separately, or grouped by twos, there is not the steady decrease in fertility with advancing inbreeding that Davenport's grouping of the data implies. Taking the data for the first two generations together, the average size of the litters was 3.7; for the next two generations there was an average of 4.0 young per litter; in the final group the litters averaged 2.7 young. Since the crossing of varieties is supposed to increase vigor and fecundity it seems strange that the F_1 and the F_2 litters in this series should contain a smaller average number of young than is found in the

normal litter of either of the varieties that were crossed (five to six young per litter).   Since crossing did not restore the normal fertility of the individuals, it would seem as if there must have been a strong tendency to sterility in each of the strains crossed. If such were the case, continued inbreeding, apparently without selection, would bring out this latent character and intensify it.

It seems rather remarkable that, of the many writers who have cited the results of the above series of experiments as proof that close inbreeding lessens fertility, not one, to my knowledge, has emphasized the fact that all of these experiments were made with hybrids and not with a pure strain.   Hybridization in itself, as many investigators have noted, often produces a most marked effect on fertility.   Some hybrids are equal, or even superior to the parent stock in fertility others are completely sterile; and among the hybrid offspring from various crosses all grades of productiveness from normal to complete sterility have been found. When hybridization increases fertility, its most marked effect is usually found in the animals of the F_1 and F_2 generations, and in later generations productiveness, as a rule, tends to decrease.

In connection with another problem, I have for several years been breeding the F_1 hybrids between the wild Norway and the albino rat, and I have also inbred various strains of 'extracted' rats, brother and sister, for several generations.   Careful records have been kept of the litter production in all of these strains. While the great majority of the F_1 hybrid females are fertile, at least 25 per cent of the F_2 females are completely sterile, and about 10 per cent of those that do breed have only one or two litters.   None of the 'extracted' strains that I have studied have even been as fertile as the inbred Albinos.   The increase of sterility and the diminution in litter size with continued inbreeding has been very marked in some of these strains, but this lessened productiveness has been due, I believe, to hybridization, and it has not been influenced by inbreeding save in as far as inbreeding has intensified the tendencies which acted unfavorably upon productiveness.   By rigid selection of only the most fertile individuals for breeding, from a large potential breeding stock, it might be possible to eliminate from the 'extracted' strains of rats

the tendency to sterility that is seemingly caused by hybridization. Such a selection was not attempted, apparently, in any of the series of experiments cited above, nor was it done in my own work with hybrid stock. The experiments of Crampe, of Ritzema-Bos, and of von Guaita show unquestionably that fertility in hybrid rats is diminished by random inbreeding, but they cannot legitimately be used to give evidence regarding the effects of inbreeding on the fertility of a pure race.

Other series of inbreeding experiments made on pure strains of rodents show that inbreeding does not necessarily lead to a marked decrease in fertility. Neither Schultze ('03) nor Copeman and Parson ('09) found inbred mice less productive than the outbred strain; Castle ('16) did not find any great decrease in fertility in various races of rats inbred for seventeen generations. In the inbreeding experiment with guinea-pigs that has been carried on for several years at the Bureau of Animal Industry in Washington, there is, to quote Popenoe ('17): "no general deterioration. While a few strains have run out, others are nearly as vigorous as are the control families."

Results comparable to the above have been obtained with other animals. It is well known that inbreeding has been used extensively, and with very favorable results, in the building up of various strains of thoroughbred horses and cattle (Wriedt, '16), and the productiveness of these strains has not been greatly lessened. In the extensive series of inbreeding experiments with Drosophila, made by Castle et al. ('06), it was found that "inbreeding probably reduces very slightly the productiveness of Drosophila, but the productiveness may be fully maintained under constant inbreeding (brother and sister) if selection is made from the more productive families. . . . . Selection has a much greater influence on fertility than inbreeding, so that selection from the most productive pairs is able to more than offset the effects of inbreeding." The effectiveness of selection in increasing the fertility within an inbred strain is shown with great clearness in Moenkhaus' ('11) experiments with Drosophila. Moenkhaus was able to establish two distinct strains, one of high and one of low fecundity, by selecting, from among the variable

offspring of the fourteenth generation of a closely inbred race, pairs of individuals showing very different degrees of productiveness and then inbreeding their descendants. Moenkhaus continued some of his lines for seventy-five generations and found that close inbreeding (brother and sister) was not deleterious either to fertility or to vigor. Hyde ('14) has found also that in certain strains of Drosophila sterility is an inherited character that is not influenced by inbreeding, and that "selection is an effective agent in controlling it."

In the present series of inbreeding experiments on the rat, the productiveness of the strain was decreased by malnutrition during the early generations, but normal fertility was restored as soon as the animals were adequately nourished. In later generations the fertility in the inbred animals was greater than that in the series of stock controls reared under similar environmental conditions. Thus even after a high degree of sterility had been introduced into the strain it was not retained in spite of the fact that close inbreeding was continued. In the later generations any tendency to sterility that appeared was evidently suppressed by selection. In the rat, as in Drosophila, selection seems a more potent factor for good than inbreeding is for evil.

During the past few years it has been shown, by a series of brilliant experiments, that characters tend to be inherited in groups and that this grouping depends upon the fact that the genetic factors involved are not segregated independently in gametogenesis, but tend to be linked together (Morgan et al., '15). In these experiments with the rat it has been found that animals that are large and vigorous when young tend to mature early, to be very productive, and to live to an advanced age. While all of these characters are influenced to a considerable degree by environmental conditions, it is evident that they must all depend to some extent upon heritable genes, since they are transmitted from generation to generation. A selection of breeding animals on the basis of size and early maturity has meant also selection for high fecundity and for characters that represent superior vigor of constitution, it would seem as if the genetic factors involved must tend to be inherited together, although they are probably not linked as are many of the genes in Drosophila.

Wentworth's ('13) experiments with Drosophila indicate that the supposed weaknesses from inbreeding are due to "the mere segregation of factors for lower vigor." Assuming that a similar segregation of these factors occurred in the inbred rats during the early generations, individuals containing the factors for 'lower vigor' were evidently eliminated by the selective action of malnutrition, and only those animals containing dominant genes for 'high vigor' were able to survive and to perpetuate their kind. Neither inbreeding nor selection is creative in its action. Selection can act on fertility only by preserving those individuals that contain genes for characters favorable to reproduction; inbreeding conserves these characters, and, to a certain extent, intensifies them. The action of both selection and inbreeding can be nullified by unfavorable conditions of environment or of nutrition which may produce a rapid deterioration in the fertility of any stock, regardless of the way in which the animals are bred.

It was shown by the work of Darwin ('78), as well as by a number of more recent experiments (Shull, '10; East and Hayes, '12; Hayes and Jones, '17), that crosses between different varieties of plants often produce hybrids that possess greater reproductive vigor than either parent stock. This result is due, according to East and Hayes ('12), to "the stimulation of vigor through heterozygosis." Inbreeding, these authors state, "tends to isolate homozygous strains which lack the physiological vigor due to heterozygosity. Decrease in vigor due to inbreeding lessens with decrease in heterozygosity and vanishes with the isolation of a completely homozygous strain." If the latter is a good strain, because of its gametic constitutional and natural inherent vigor, it is "ready to stand up forever under constant inbreeding."

The results obtained in these inbreeding experiments with the rat accord with the theory of East and Hayes to some extent. The effects of inbreeding on the fertility and on the vigor of the rats were obscured in the early generations by the action of malnutrition, but it would appear that the animals lost very little of their constitutional vigor during this time, since adequate nutrition soon restored the normal productiveness of the strain and its general vigor as well. Apparently at about the tenth generation,

the inbred rats became sufficiently homozygous for vigor to become fairly constant. Beyond the point, as the data show, there was little variation in the fertility or in the longevity of the animals up to the twenty-fifth generation. Selection and favorable environment kept the strain at a point of high productiveness, but, under the conditions of the experiment, they did not increase vigor beyond the stage which was reached at the tenth generation. As already stated, no attempt was made in the course of these experiments to influence fertility by selecting breeding animals from large or from small litters. Whether selection can act in the rat, as it does in Drosophila, and produce strains of high and of low productiveness within a line that has been inbred for many generations is a problem for the future. As the strain has been very fertile for many generations it seems very improbable that any sudden loss in fertility will occur in the future, unless sterility appears as a mutation which cannot be eliminated by selection.

While corresponding records for the two inbred series (A,B) are in close agreement, there are, nevertheless, differences between the series that have persisted from the very beginning. Female A, one of the two females with which the experiment were started, showed a relatively high degree of fertility since she gave birth to five litters, containing thirty-five young, before she was killed at the age of one year: female B, a sister of female A, cast only one litter of five young, although she was paired continuously for several months and appeared to be in good physical condition. The two litter brothers with which these females were paired showed no marked differences in size or in vigor. The rats of the A series (which were descended from female A) were, as the records show, somewhat more fertile than the rats of the B series (the descendants of female B), and they also tended to mature earlier and to live longer. The differences found were not very marked in any case, and they might well be ignored were it not for the fact that in all of the characters noted the animals of the A series were superior to those of B series. Environment cannot be held accountable for these differences, since the two series of inbreds were kept constantly under similar conditions of

light, of temperature, and of nutrition. Although the two series were descended from the same ancestral stock, apparently there was an inherent difference in the gametic constitution of the two pairs of rats with which the experiment was started, which persisted from generation to generation and produced the effects noted.

While the inbred strain of rats that has been developed in the course of these experiments is seemingly superior to the average run of stock Albinos in body size, in fertility, and in longevity, I do not claim that this superiority is due solely to the fact that the animals were inbred, neither do I wish to assert that, in general, inbreeding is better than outbreeding for building up and for maintaining the general vigor of a race. The two forms of breeding are not mutually exclusive: each has its merits, and the one should supplement the other to bring out the best in any stock. The favorable results that have been obtained in these experiments have been achieved through the constant selection of only the best animals from a larger number available for breeding purposes and by keeping the environmental conditions as uniform and as favorable as it was possible to make them. These experiments have fully demonstrated, I think, that even in mammals the closest form of inbreeding possible, i.e., the mating of brother and sister from the same litter, is not necessarily injurious either to the fertility or to the constitutional vigor of a race even when continued for many generations. Success or failure in inbreeding experiments depends chiefly, it would seem, on the character of the stock that is inbred, on the manner in which the breeding animals are selected, and on the environmental conditions under which the animals are reared. There is no warrant, therefore, either in theory or in fact, for the dogmatic assertion of Kraemer ('13) that: "continued inbreeding must always result in weakened constitution, through its own influence."

4. SUMMARY

1. The present paper gives data showing the fertility, the time of puberty, and the longevity in two series of albino rats (A,B) that were inbred, litter brother and sister, for twenty-five generations.

2. Data given for the A series of inbreds comprise 1752 litters containing 13,116 individuals, or an average of 7.5 young per litter (table 1); records for the B series of inbreds include 1656 litters having a total of 12,336 members, or an average of 7.4 young per litter (table 2). The two series combined comprise a total of 3408 litters which contained 25,452 individuals. For the entire strain the average size of the litter was 7.5 young.

3. In any litter series of albino rats, whether the animals are inbred or outbred, the first litter cast is the smallest of the series, as a rule; the second litter is the largest; while the third and fourth litters are about the same size and a little smaller than the second litter.

4. The size of any litter cast depends chiefly on the age and physical condition of the female, and is not affected by the relatedness or the unrelatedness of the parents.

5. A comparison of the data for the inbred strain with data for litter size obtained from a series of stock Albinos reared under the same environmental conditions as the inbred strain shows that each litter of the stock series was relatively smaller than the corresponding litter in the inbred group. For the entire series of 424 stock litters the average size was 6.7 young per litter. This average is 0.8 less than the average for litter size in the inbred strain (table 7).

6. In the A series of inbreds the range in litter size was from one to seventeen; in the B series it was from two to fifteen. In both series the most frequent litter size was seven (table 8).

7. In the early generations of these inbred rats malnutrition greatly delayed the time of puberty in the animals. In the later generations, under favorable nutritive conditions, the animals bred at a relatively early age.

8. While the records give no definite information regarding the number of sterile animals in the inbred strain, they show clearly that inbreeding did not decrease the productiveness of the animals. Of the 954 females that were used for breeding, 653, or 68.5 per cent cast the required number of four litters. Where partial sterility occurred in apparently healthy females it was found to be due to a diseased condition of the reproductive organs.

9. The constitutional vigor of these rats was apparently not impaired to any extent by inbreeding. Only two kinds of malformations were found in the animals of the inbred strain after food conditions were improved: one individual was born tailless and about a dozen individuals lacked one or both eyeballs. Both of these defects occur in outbred stock Albinos and neither appears to be heritable.

10. Under the conditions of these experiments the span of life in both the males and females in each of the inbred series was increased. The records show that inbred males tended to live longer than did inbred females: a reversed relation was found in the animals of the control series. In the inbred colony as a whole, the females seemed to be longer lived than the males and they were less susceptible to disease at all ages.

11. According to the behavior tests that were made, inbred Albinos are slower, less active, more timid and nervous, and somewhat more savage than stock Albinos that are outbred.

12. High fecundity, early sexual maturity, and vigorous growth are characters that seemed to be inherited as a group in the inbred strain of rats. It seems probable that the genetic factors on which these characters depend do not segregate independently, but tend to combine in gametogenesis.

13. The animals of the A series were slightly more fertile than the animals of the B series, they attained sexual maturity earlier, as a rule, and they lived longer. These differences probably depended in some way on a dissimilarity in the gametic constitution of the two pairs of individuals with which the experiments were started.

14. The results obtained in these experiments do not accord with the general view regarding the effects of inbreeding, since they indicate that inbreeding per se is not necessarily inimical either to fertility or to vigor. Success or failure in any series of inbreeding experiments would seem to depend on the character of the stock that is inbred, on the manner in which breeding animals are selected, and on the environmental conditions under which the animals are reared.

THE AMOUNT OF BOTTOM MATERIAL INGESTED BY HOLOTHURIANS (STICHOPUS)[1]

W. J. CROZIER

Bermuda Biological Station for Research

TWO CHARTS

I. It has repeatedly been suggested that animals which obtain their food by the ingestion of sandy or muddy bottom materials harboring minute organisms may exert an important influence upon the local topography of the sea-floor. No attempts seem, however, to have been directed toward estimating the amounts of material which may actually be 'worked over' in this way. Large holothurians, such as Stichopus, Actinopyga, and to a lesser degree species of Holothuria, are found in great profusion upon shallow littoral bottoms among 'coral' reef islands in the warmer seas, and Gardiner, Verrill, Mayer, and other observers agree in attributing to them a particular significance for the disintegration and scattering of calcareous bottom deposits. These relatively huge holothurians are very plentiful, they usually contain a considerable amount of sand, and the bottom is in places thickly strewn with their castings; it is very natural to suppose that the feeding activities of these animals may have an effect upon the sea-bottom not unlike that, so carefully described by Darwin, which earthworms produce in the soil. Thus it might be considered that the production of many fine sand particles would be facilitated by mutual grinding in the intestinal tract, and that the intestinal juices might also aid in the solution of calcareous fragments. From this point of view, as well as with the object of securing data for use in a study of digestion in these holothurians, I have endeavored to estimate the amount of bottom material which may be passed through the digestive

[1] Contributions from the Bermuda Biological Station for Research. No. 88.

tract of the large Stichopus moebii Semper, which is so abundant on grass-free bottoms of sandy mud about the Bermuda Islands.

An adequate estimate of this nature requires information upon the following points: 1) the maximum amount of material usually contained in the gut of Stichopus of different sizes; 2) the frequency with which holothurians of different sizes feed; 3) the frequency-distribution of sizes in the Stichopus population; 4) the actual numerical abundance of Stichopus in regions of known area.

It is necessary to explain, in connection with 1) and 2), that Stichopus is very convenient for a study of this kind, since as a rule the intestine is filled, completely, before defecation is begun; and that an appreciable interval elapses between defecation and subsequent feeding, so that the gut is for a time entirely, or almost entirely, empty of solid contents. Feeding may begin before the intestine has been emptied completely, but each filling of the gut seems to be handled as a unit; characteristically, the whole contents of the intestine are voided rather rapidly and in a continuous mass. Hence the long castings which are to be seen upon the bottoms where Stichopus lives.

These observations were confined to shallow-water situations, and the 'quantitative' data refer to a season (September–November, 1917) when the temperature of the water was between 17° and 24°C., usually below 20°C. Stichopus occurs down to depths of 8 fathoms, and possibly more. It is almost certainly more abundant, however, in shallow water along the shore.

' II. 1. The amount of sand held in the intestine of Stichopus just before evacuation is begun appears to be somewhat variable, in animals of about the same size, even when they are collected in the same locality. Large numbers of them were opened, at various hours of the day and at different stages of the tide, and from the examination of the alimentary tracts of these individuals thirty-nine were selected which seemed to be filled to the maximal extent. The gut contents, in each of these cases, were removed to a finger bowl, well washed with rain water, the sand filtered off, dried in the sun, and weighed. All of these animals were obtained upon one kind of bottom, near Agar's Island. Figure 1,

A, shows the relation obtained between the length of a Stichopus and what may be called the maximal, or 'full,' intestinal capacity. The errors involved in weighing the contained mud are probably no greater than those incidental to the measurement of the length of a Stichopus.   The intestine in the instances considered 'full' was tightly packed with sand, as was also the oesophagus and buccal chamber.   In the region of the stomach proper, at least in its anterior half, the material was less closely compacted.
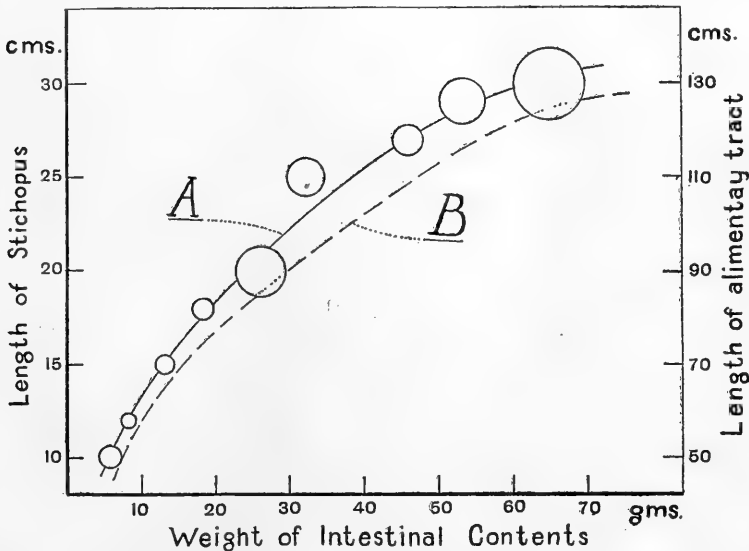
Fig. 1   A (scale to the left)—relation between length of Stichopus and the average weight of the washed, air dried, contents of the 'full' alimentary tract. B (scale to the right)—Same for the length of the intestine (from figure 2).

The several regions of the alimentary tract of Stichopus, it may be mentioned incidentally, are more sharply delimited than text-book descriptions of holothurian anatomy would lead one to sup-pose.   The short white buccal chamber is followed by a very thin-walled, highly contractile, oesophagus, which is more or less heavily pigmented by a substance belonging to the 'echinochrome' or 'antedonin' group.   This oesophagus appears to exert a suctorial function; but the activities of the different segments of the alimentary tract may be better considered in a subsequent

article.   In the pigmented oesophagus lies the region at which the anterior connections of the gut are ruptured in the process of visceral autotomy.   The oesophagus leads into the stomach, which occupies the remainder of the first ('dorsal') loop of the gut, passing posteriorly into the reflexed intestine.   The intestine itself is white, and of uniform diameter throughout its length down to the sphincter constriction at the point of entrance into the cloaca.   The stomach, two or three times the diameter of the intestine, contains yellowish or orange digestive fluid, and by peristaltic movements mixes this fluid with the ingested mud. The material which is passed on into the intestine is there compacted and for several hours undergoes a process of segmentation before being voided in a more or less continuous mass.

The total length of the gut increases rapidly with the size of the Stichopus (figure 2), and the maximal capacity is less than proportional to the cube of this length (figure 1, B).   The length of the alimentary tract was estimated in a number of 'full' individuals, as the intestine is very quickly shortened by contraction when cut from 'empty' specimens.   The direct proportionality between gut-length and length of individual (figure 2) seems to show that the judgment based on the 'full' condition (employed in the construction of figure 1, A,) was sufficiently accurate.   The fact that the stomach is never entirely filled with sand, but always contains a fair volume of digestive juices, probably accounts for the deviation from expectation regarding the relation between length and gut-capacity (figure 1, B,); as it is, the ratio—(dry weight of maximal contents): (cube of the length of gut)—decreases with increasing length of animal.

These determinations show that when filled to its maximum the gut of an average Stichopus of ordinary length (27 cms.) contains about 46 gms. (dry weight) of bottom material; this is mainly composed of calcareous fragments of various kinds.   If it 'ate' but once in the twenty-four hours, such an individual would pass through its intestine, and deposit in the form of castings, something like 1.4 kilos (dry weight) of bottom material in one month, at this time of year (November).   This estimate is, as a matter of fact, much too low.

2. It is rather difficult to determine exactly the number of times per day that these holothurians fill the intestine.   Laboratory feeding experiments are quite useless, as the animals usually will not eat when confined in vessels having vertical walls up which they may creep.   The castings do not retain, in the field, a recognizable form for more than several hours, so that little help can be had from that quarter.   Moreover, these animals move about to an extent which one would scarcely predict. When viewed from the shore, they seem ponderously sluggish,—
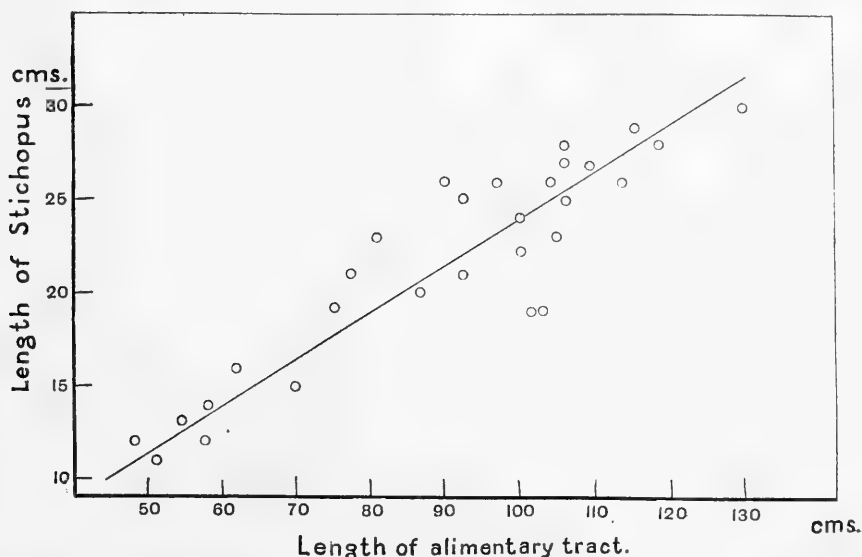
Fig. 2   Relation between length of animal and length of gut, in Stichopus moebii.

a part of the bottom,—yet they do in fact exhibit quite decided migratory movements, particularly in a vertical direction, in appropriate places.   Several attempts to get Stichopus to feed upon a substratum prepared by mixing lamp-black with the natural mud, in order that the mud might be recognizable if swallowed, were unsuccessful.   The examination of normal intestinal contents plainly showed that the periods of emptying were not characteristically coincident with tidal events.   Attempts to discover the existence of any other form of regularity

in the feeding activity of Stichopus led to the discovery of a somewhat surprising condition.

Three fairly representative localities were chosen for detailed observation, the places in question being sufficiently near together so that they could be inspected in rapid succession. The first was a shallow, well protected, bight in Crow Lane, immediately to the north of Saltus Island; the second, along the east shore of Agar's Island; the third, along the north side of a narrow ridge of rock projecting westward from an island situated at the mouth of Fairyland Creek. Stichopus was abundant at each of these stations. When the contents of the alimentary tract of a dozen or more specimens from one of these places were examined, it was found that as a rule the great majority of the individuals were in precisely the same condition as regards the presence of ingested mud. At certain times of the day all of the individuals in any one locality were filled to an identical degree. The Stichopus at the different stations were, however, in very different conditions as regards contained mud. Thus, on several occasions each of 12 Stichopus taken at 8:30 A.M. near Saltus Island was found with the gut completely empty; while those at Agar's Island and at the mouth of Fairyland Creek were uniformly 'one half full,' the posterior portion of the intestine being empty, although they were collected only a few minutes after the first lot had been obtained. Repeated collections of this sort, at different hours of the day, convinced me that all the holothurians in any one locality were usually to be found with the intestine filled to about the same extent. The reason for this curious uniformity I am not yet in a position to discuss, but it is a condition very useful for the purposes of the problem treated in the present paper.

The next step consisted in determining the rapidity with which the intestine of Stichopus is filled and emptied. Observations were made in the three localities previously mentioned; the work was of necessity restricted to periods of fine weather. During storms the holothurians do not remain near the surface, but in exposed places, retreat to a depth of several fathoms. They

also move into deeper water at nightfall, and gradually creep up to near the surface during the morning hours.

I quote from a record of observations made during a single day; these records are typical of my findings upon other occasions.

December 12, 1917

Agar's Island	Rocky spit near Fairy-land Creek	Bay north of Saltus Island
7 : 05 A.M. No castings visible	8 :00 A.M. No castings visible	8 : 30 A.M. No castings apparent; 5 animals opened had the intestine well filled.
7:30–8:00. Castings by 10 of the 12 inspected animals.	10:30–11:00. Of 63 animals, 60 in the process of defecating.	10:00. Castings plentiful; of 6 opened, only 1 animal had sand in the gut.
9:30. 10 full, 1 filling, 1 emptying, 1 empty (13 opened).	4:30 P.M. Of 48 animals, all had castings near them or were defecating.	2:00 P.M. No castings; 11 opened; all were full or at least (2 cases) $\frac{2}{3}$ full.
11:00. Of 8 animals, 4 had begun castings.		4:45. Of 28 animals, all exhibited castings.
11:30. 8 dissected; 5 empty, 2 well filled, 1 with oesophagus and stomach full.		
4:30 P.M. No castings visible; 4 examined had the intestine well filled.		

It follows that these holothurians were probably engaged in filling the intestine at least three times in the twenty-four hours. During daylight, the process of filling and emptying appears to occupy, at a temperature of about 20°C., a period of about 5–6 hours.   In utilizing these results for further calculations, I have considered that in any given littoral situation frequented by Stichopus each animal fills the gut twice each day.   It seems reasonable to make this restriction owing, 1) to the fact that at night the holothurians retreat into deeper water, and 2) to the fact that in stormy weather they remain well below the water surface, except in protected places; this restriction is probably of a character to diminish, rather than to enhance, the calculated tilling of the sea-bottom by Stichopus.   The observations on feeding showed that animals of all sizes between 10 and 30 cm. length fill the intestine with about the same frequency.

Estimates of the quantities of bottom material swallowed by Stichopus moebii at certain localities in Bermuda. The calculations based on the number of Stichopus present in the areas measured, their average size, and the assumption that they eat twice each day

LOCALITY	AREA	NUMBER OF STICHOPUS*	KILOS DAY EATEN	GRAMS PER SQUARE METER PER DAY
	feet			
Agar's Island................	1000 × 35	97	7.5	2.7
Spit Head...................	100 × 25	37	6.5	28.0
Marshall Island.............	100 × 50	675	13.5	29.0
Harrington Sound...........	75 × 50	67	2.9	8.3
Fairyland Creek.............	100 × 15	63	5.0	25.0

* The number seems *fairly* constant in any one locality; for example, in the small area studied on the south side of Marshall Island, between 600 and 700 young Stichopus (between 10 and 18 cms. length) were constantly present from August to November. There is a very decided tendency for all those in one locality to be of about the same general size.

III. Employing the reasonable, and I believe exceedingly moderate, assumption that, on the average, Stichopus fills the intestine two times each day throughout the year, and taking into account the average size of the individuals locally concerned (with the aid of fig. 1, A), the calculations exhibited in table 1 were made in order to obtain some idea of the magnitude of the effects which may properly be ascribed to the feeding activities of Stichopus. It is further assumed that the specific weight of the bottom-material is the same in the different localities mentioned, an assumption which is well within the limit of error of other parts of the calculation. I have no desire to convey the impression that these figures (table 1) possess any notable precision, but I am unable to see that they could be made more exact without the expenditure of an unprofitable amount of energy.

It will be noted that any inadequacy attaching to these estimates is in all probability such as to make the results too small rather than too large. Yet the average figures appear to be of considerable magnitude. It seems that for each square meter of bottom, in the localities studied, between 2.7 and 29 gms. of calcareous deposits are passed through the gut of Stichopus each day. This leads to the conclusion that on the average, in these

places, something like 6.8 kilos (dry weight) per square meter per year is eaten by Stichopus. The amount really concerned cannot very well be much less; it may be one and one half to two times as great.

If we attempt to figure the weight of bottom material eaten by Stichopus in one of the partially enclosed sounds at Bermuda, such as Harrington Sound, which has a superficial area of about 1.7 square miles, we find that probably not less than 500,000 kilos, or say between 500 and 1000 tons, of bottom substance passes through the intestine of this holothurian each year. This calculation, necessarily of a rough order, is based on the assumption that Stichopus is about as frequent in Harrington Sound as in the average of places listed in table 1, and that the holothurians eat but twice each day; these assumptions are admittedly rough, but their respective inadequacies probably tend to counteract each other.

IV. The geological importance of the feeding activities of Stichopus is determined by the magnitude of the changes which may be produced by any or all of the following influences: 1) in moving about, the holothurians may carry from place to place some portion of the bottom deposits; as subsequently liberated in castings, the ingested material may thus be carried near to the water surface, exposed to wave action, and redistributed over a new section of the bottom; 2) the mutual attrition of calcareous fragments, especially when the ingested mass is undergoing segmentation in the intestine, may produce in a mechanical way particles of a fine degree of subdivision; 3) the intestinal fluids may dissolve part of the calcareous material; if subsequently precipitated in flocculent form, upon being expelled into the sea, this material would assist in the accumulation of ooze.

The first of these three factors is undoubtedly of some consequence. The castings are readily broken up by currents, even at a depth of several fathoms. The mucus which surrounds and impregnates the ejected mass may have an action, as a protective colloid, in assisting the dispersal of the finer particles by the water, but this action can only be of a brief and temporary character, since the slime is soluble in sea water.

The second influence has, I think, been in the past over rated. Microscopic examination shows that delicate bryozoan skeletons, sometimes in pieces 5 mm. x 2 mm., pass through the intestine apparently unscathed, as do also bits of echinoid spines 5–6 mm. in length. There is no detectable increase in the amount of finely ground material in the last centimeter of the intestine as compared with that in the oesophagus. I made a number of tests in which the contents of the oesophagus, or of the buccal chamber, were compared with those of the last portion of the intestine, by suspending the mud in tall cylinders of water; these cylinders were shaken well, and the contents allowed to sediment. The proportion of fine material to that of coarser grade was in all cases the same. When opening the intestine of a Stichopus, one gets the impression that the contents are more finely divided than are those of the oesophagus, because most of the smaller particles are on the outside of the densely compacted mass. Nevertheless, some grinding may take place, but this factor seems to be relatively unimportant.

The partial solution of calcareous fragments may be of greater significance. The yellow fluid contained in the stomach of an 'empty' Stichopus gives with indicators an apparent acidity of $P_H = 5.0$–6.5. Fluid obtained by centrifuging the stomach contents of animals engaged in feeding showed acidities varying from 4.8 to 5.5, the latter being most common. There seems to be an active secretion of acid at the time of feeding. This acidity is adequate to dissolve some calcium carbonate, and is in fact greater than that of the rain water which forms stalactites in the limestone caves at Bermuda; the freshly fallen rain water is at about $P_H = 6.0$, while that caught directly as it dripped from the tip of stalactites in several caves which I investigated was at about $P_H = 7.9$–8.0. This seems to be the most important influence which Stichopus exerts, geologically; namely, the solution of a small amount of calcium carbonate, which is probably soon precipitated again when the intestinal contents are ejected into an alkaline sea water ($P_H = 8.1$–8.2). In this way these holothurians may have played a not inconsiderable part in the excavation of lagoons, and in the formation of muddy deposits,—

even though they do not devour corals as Darwin at first believed from the reports made to him and from the supposed masticatory function of the stone-ring.

SUMMARY

1. A preliminary estimation has been made of the amount of bottom material (mainly calcareous) deposited in littoral situations about the Bermuda Islands which may be passed through the intestine of the large holothurian, Stichopus moebii Semper.

2. This estimate is based upon the fact that it is possible to obtain a fairly accurate idea of the rate of feeding in Stichopus, and of the maximal contents of the gut in individuals of different sizes.

3. In certain typical areas frequented by this species the amount of bottom material passing through the intestine of Stichopus is roughly 6 to 7 kilos (dry weight) per square meter per year.

4. It is estimated that in the enclosed sink Harrington Sound the amount of bottom deposit annually eaten by Stichopus is perhaps 500 to 1000 tons.

5. The fluid stomach contents of Stichopus are sufficiently acid to dissolve some calcium carbonate. The mutual attrition of particles in the intestine is probably of small significance for the formation of finely divided particles.[2]

Pembroke, Bermuda, January 5, 1918.
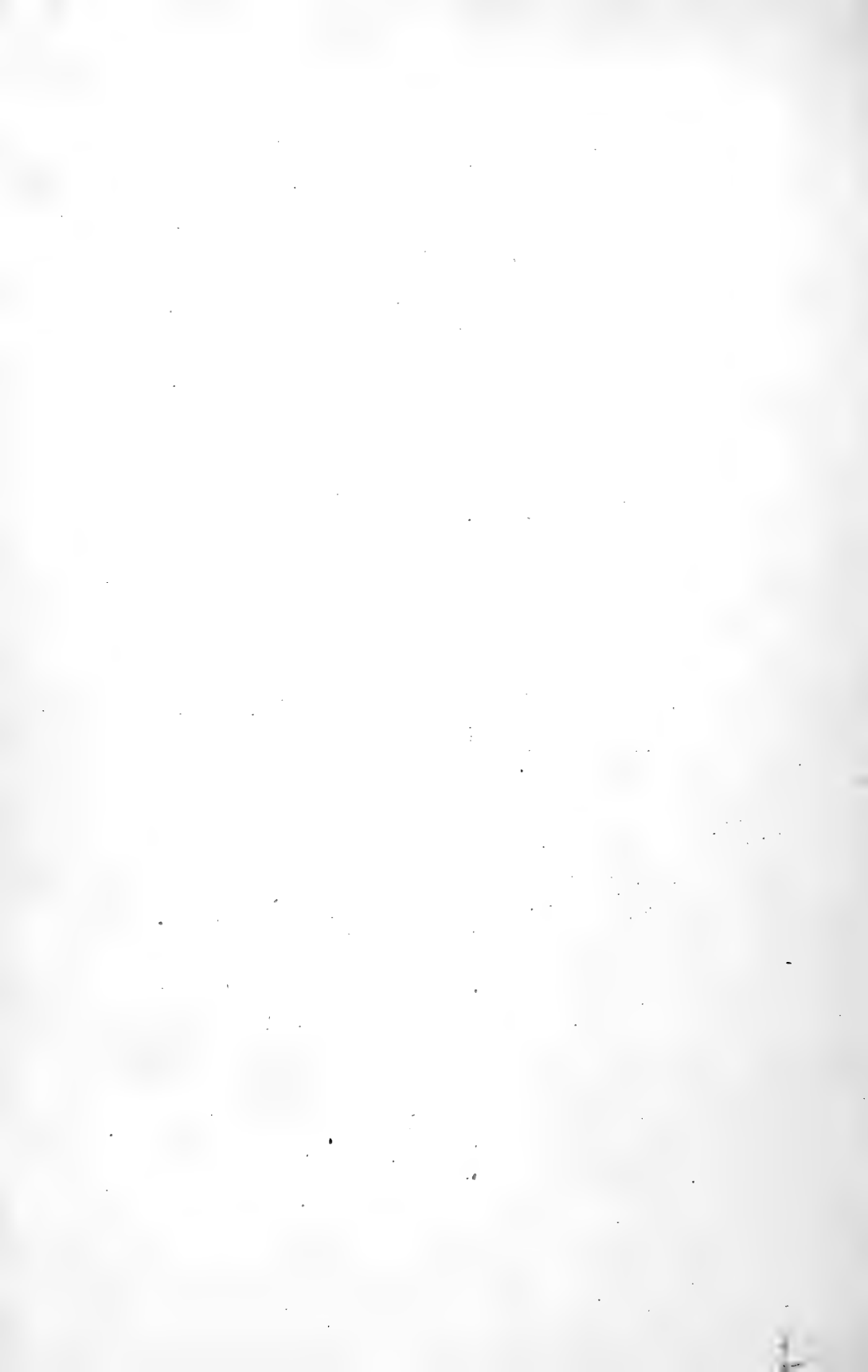
HYBRIDS BETWEEN FUNDULUS AND MACKEREL

A STUDY OF PATERNAL HEREDITY IN HETEROGENIC HYBRIDS

H. H. NEWMAN

Hull Zoological Laboratory, University of Chicago

FOUR FIGURES

I. INTRODUCTION

The present status of heterogenic hybridology in animals

Experiments in hererogenic hybridization of animals have been performed largely upon two groups, Echinoids and Teleosts. A much larger amount of attention has been paid to the former than to the latter group, although it now seems certain that the Teleosts in a number of important respects are more favorable than the Echinoids for this kind of work. The list of investigators who have contributed to the literature of hybridization among the Echinoids is imposing, including as it does such names as Boveri, Seeliger, Morgan, Vernon, Driesch, Delage, Steinbruck, Loeb, Doncaster, Peter, Fischel, Herbst, Godlewski, Kupelwieser, Hogedoorn, King, Moore, Tennant, McBride, Fuchs, Debaisieux, Shearer, and DeMorgan. Only a few, largely Americans, have studied hybridization in Teleosts and the work has been comparatively superficial. Only the following seven or eight investigators have contributed to this field: Appellöf, Bancroft, Hertwig, G. and P. Loeb, Moenkhaus, Morris and Newman. It is my conviction that had the same amount of attention been applied to Teleost hybridization as has been given to that of Echinoids, we would have a vastly better understanding of the truth about heterogenic hybridization phenomena that we now have.

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The hybrid situation in Echinoids

A close comparison of the results of hybridization in Echinoids with that of Teleosts has served to place the whole matter in a new light and to emphasize the need of further work on Teleosts. It seems necessary in making such a comparison to show graphically the interrelations of the species that have been crossed. The following table gives in italics the genera of Echinoids used in all of the various experiments, together with an outline of their classification (table 1).

TABLE 1

GENUS	FAMILY	SUBORDER	ORDER	SUBCLASS
Arbacia	Arbaciidae	Arbacina		
Echinus *Hipponoe* *Toxopneustes*	Triplechinidae	Echinina	Diademoida	Regularia
Strongylocentrotus *Sphaerechinus*	Strongylocentroidae			
Echinocardium	Spatangidae	Sternata	Atelostomata	Irregularia

A goodly number of investigators have crossed Strongylocentrotus and Sphaerechinus, using a number of species of each genus. Such crosses are of merely intergeneric width and are hardly to be classed as heterogenic.

Interfamily crosses have been carried out between Echinus of the family Triplechinidae and Sphaerechinus of the family of Strongylocentrotidae, and there is little difference of opinion as to the evidences of paternal heredity. Inter-sub-order crosses between Arbacia of the suborder Arbacina and members of the suborder Echinina have given less definite results as to the hereditary effect of the foreign sperm, Driesch stating that the larvae were pure maternal and Fischel that the paternal influence is shown in form, size, pigment, and skeleton. Neither author was able to rear the larvae far enough to study very definite characters.

Only one cross of greater than suborder width has been made within the bounds of the class Echinoidea and that is of subclass width.   McBride and Fuchs have independently crossed a representative of the subclass Regularia with one of the subclass Irregularia, using Echinus and Echinocardium.   The percentage of fertilizations is so low and the larvae are so unhealthy that no definite conclusions were reached by either author.

By means of chemical aids to insemination, 'crosses' of interclass width have been made between Echinoidea eggs and the sperm of Astereridea, Holothuroidea, Ophiuroidea and Crinoidea. Though extremely few larvae resulted—and these were decidedly abnormal—they were described as pure material.   By the same chemical means Echinoid eggs have been inseminated with the sperm of Mollusca and Annelida, and occasional stunted larvae have resulted that also are said to be pure maternal.   These interphylum 'crosses' mark the extreme limit of heterogeneity in hybridization, if indeed they are hybrid phenomena at all. One step further and we would have crosses of subkingdom value (Protozoa $\times$ Metazoa) or even of kingdom value (plants $\times$ animals), which would amount to a reductio ad absurdum.

It must be borne in mind that, in Echinoids, 'crosses' of greater than suborder width are impossible in nature and can be made only by the use of chemical agents that serve to break down the normal incompatibility of the heterogenous germinal materials, and may well play a rôle analogous to that of parthenogenetic agents.

'Crosses' of greater than suborder width are described as being purely maternal, although the criteria for this judgment are extremely doubtful since the embryo and larvae reared are highly pathological.   No paternal heredity is to be expected in view of the fact that the foreign chromatin forced into the egg unnaturally, utterly fails to coöperate in cleavage or in more general cell metabolism, but remains an inert mass till gradually eliminated by absorption.   An exception appears to exist in the case of the cross between Echinus and Antedon, in which Godlewski claims a normal behavior of the paternal chromosomes, except that

the latter come to be indistinguishable from Echinus chromosomes; yet the larvae are pure maternal in so far as they are anything definite. In these wide 'crosses' it appears reasonable to conclude that the foreign sperm at least assists in the initiation of development, duplicating in a sense the rôle of parthenogenetic agents. According to Loeb: "The egg behaves exactly as we should expect from the fact that the spermatozoön removes only certain obstacles for the development of the egg, but does not cause its development by carrying any activating enzyme."

It may be concluded, then, that in Echinoidea crosses of generic and family width exhibit paternal heredity, that when Echinoids are 'crossed' with other classes or other phyla that heredity is not concerned, but merely initiation of development. The really interesting and critical phases of Echinoid hybridization lie between these extremes: in crosses of subordinal, ordinal, and subclass width, and there are, unfortunately, very few data on this point.

A cross of subordinal width was studied by Driesch and by Herbst. Their results and conclusions are diametrically opposed; the former concluding that the hybrid larvae were purely maternal and the latter that the paternal influence was seen in several characters. More work is needed upon crosses of this width. A cross of subclass width was made by McBride and repeated by Fuchs with results quite different in the two cases, and it seems certain from what these authors say that the larvae are so few and so abnormal that little conclusive evidence as to heredity is available.

In conclusion it may be said that the results of hybridization experiments of greater than interfamily width among the Echinoids have been entirely inconclusive, and it would appear necessary to transfer our attention to Teleost material if we wish for any approach to a definite answer as to the question whether heterogenic hybrids show paternal heredity or are merely parthenogenetic in character.

The hybrid situation in Teleosts as compared with that in Echinoids

Taxonomically the group Teleostei is of lower value than the group Echinoidea, the former being merely an order of the sub-class Teleostomi of the class Pisces, while the latter is a class on a par with the class Pisces. A group of Echinoidea correlative with the order Teleostei is the order Diademoida of the sub-class Regularia. A strict comparison of hybrid phenomena would therefore be limited within the confines of these two orders, Diademoida and Teleostei. As a matter of fact, only one Echinoid cross has been made that exceeds the limits of the Diademoida.

No one has as yet succeeded in crossing a Teleost with any of the Ganoid orders of Teleostomi nor with any other subclass of Pisces, much less with members of other classes of vertebrates or with invertebrate phyla.   By the use of appropriate chemicals it might conceivably be possible to inseminate Teleost eggs with other than Teleost sperm, but my impression is that the micro-pyle would be too much of an obstacle for a type of sperm very different from that of a Teleost.   Even if a foreign spermatozoön could be forced past the micropyle barrier, it would doubtless be unable to initiate development for the same reasons that the several sorts of parthenogenetic agents, that have been extensively tried upon Teleost eggs, have failed to bring about any develop-mental result; for Teleost eggs, unlike Echinoid eggs, are extreme-ly refractory to parthenogenetic agents and would therefore probably fail to respond to foreign sperm. Hybridization ex-periments among Teleosts are, therefore, confined entirely within the order and at most will be of subordinal width.   A very large number of crosses of subordinal, of family, and of generic width have been made.   A companion of the following table (table 2), which represents the classification of the genera of Teleosts, with table 1 will be of interest.

A survey of this table shows that nearly half of the genera used are members of the large suborder Acanthopterygii, just as most of the Echinoid genera used in crosses belong to the suborder Echinina.   The four suborders in the table have been crossed in a great many ways and with varying degrees of success.   At

least as wide a cross as any possible within the bounds of the
genera given in this table is that between the first and the last
genera, Fundulus and Scomber.   In general it may be said that
any Teleost will cross with any other with the exception of vivi-
parous species, and those with peculiar breeding or brooding
habits, such as the pipe-fishes, where secondary obstacles to cross
insemination present themselves.   It is also worthy of note that
no artificial aid to insemination is necessary in any cross.

In the case of crosses between closely allied species, as, for
instance the various species of Fundulus (F. heteroclitus, F. ma-
jalis, F. diaphanus), in which paternal heredity is as obvious in
many ways as is maternal, in that paternal characters are often

TABLE 2

Genera of Teleostei used in hybrid experiments

GENUS	FAMILY	SUBORDER
Fundulus ⎫ Cyprinodon ⎭	Cyprinodontidae	Haplomi
Gasterosteus ⎫ Apeltes ⎭	Gasterosteidae	Cateasteomi
Menidia Pronotus	Atherinidae ⎫ Stromatidae ⎭	Percosoces
Morone	Serraninae ⎤	
Stenotomus	Sparidae ⎥	
Tautogolabrus ⎫ Tautoga ⎭	Labridae ⎥	Acanthopterygii
Scomber	Scombridae ⎦	

dominant over maternal.   The same is clearly true for crosses
between two genera of the same family as when the genus Gas-
terosteus is crossed with Apeltes, both genera belonging to the
family Gasterosteidae.   In this case a large percentage of healthy
hybrid larvae hatch and they are by no means pure maternal.
Crosses of interfamily width such as that between representatives
of the different families of Acanthopterygii give similar results, in
so far as success in development and strength of paternal heredity
are concerned, as do crosses of suborder width and need not be
considered at length.   As a very typical case of heterogenic
hybridization of suborder width among the Teleosts I have
chosen that between Fundulus heteroclitus of the suborder

Haplomi, and Scomber scombrus of the suborder Acanthopterygii. This cross between mud-minnow and mackerel is an excellent one for our purposes because it shows in the clearest way what many other crosses show, but show in a less obvious way.

One reason for making an intensive study of one particular heterogenic cross was because I have felt that certain incorrect conclusions as to the nature of heterogenic Teleost hybrids had been published by well-known writers. Moenkhaus holds that the development of these hybrids is pure maternal and that the cleavage rate of the hybrid egg is unaltered by the foreign sperm even if the cleavage rate of the paternal species is much slower or much more rapid than that of the maternal species. I have shown that this is incorrect by the use of more exact methods of comparison. There is an acceleration of cleavage and development, accompanied by a heightened vigor when two very closely allied species are crossed. In any crosses except those of very closely allied species the rate of cleavage and subsequent development is retarded and more or less abnormal (subnormal) larvae result. This does not depend directly on the distance of the cross, for sometimes crosses of suborder width show less retardation and a higher viability than crosses of generic width.

Loeb, on the basis of certain rather unfortunately chosen crosses, supports Moenkhaus in his statement that cleavage rate and that of subsequent development is pure maternal and goes farther in claiming that heterogenic hybrids are pure maternal in their hereditary characters. He thinks the hybrid larvae, which in all of his experiments appeared to be quite unhealthy, are merely pure maternal or parthenogenetic larvae that are poisoned by the presence of materials brought in by the sperm. The argument in favor of the pure maternal character of development, which implies parthenogenesis, is that "if the development of the egg were caused by an enzyme carried into the egg by a spermatozoön, developing eggs should be accelerated by a spermatozoön of a species developing at a faster rate." This conclusion does not appear to me to follow at all, for we must not forget the high specificity of enzymes. An enzyme that is capable of setting up a high rate of activity in one species of proto-

plasm would not be expected to activate so readily another species of protoplasm. It might conceivably retard development beyond the normal rate even in a slowly developing egg and still play the typical rôle of sperm materials in heredity. This is, I believe, exactly what happens in all heterogenic hybrids, for they all exhibit more or less pronounced evidences of early and long-continued retardation. That the sperm actually do coöperate in development even in suborder crosses is shown by the unmistakable cases of paternal heredity. It is inconceivable that a foreign sperm could function in heredity without effective functioning in development, and any hybrid in which paternal heredity is demonstrated is neither a parthenogenetic individual nor pure maternal.

It is just exactly this point that the present experiments demonstrate, to my mind at least, beyond controversy. I have selected the Fundulus × Mackerel cross out of nearly one hundred crosses that I have personally made among the Teleosts because it is especially favorable. In no sense, however, is it exceptional or peculiar in character, merely a little clearer and more diagrammatic than others that might have been chosen. Any one of half a dozen other crosses would have done nearly as well.

II. EXPERIMENTAL

Differences between adults of Fundulus heteroclitus and Scomber scombrus

Fundulus and the mackerel are sharply contrasted in all of their adult characters as might be expected in representatives of different suborders. They differ radically in habitat, ecological relations, breeding habits, eggs, and larvae. Fundulus is a minnow with shore-feeding habits, is found in both salt and brackish water, is tolerant of foulness in water and to low oxygen and high CO_2 concentration. The large eggs ($2\frac{1}{2}$ mm. in diameter) are laid during a clasping act on the part of the male which insures fertilization of the eggs with a minimum expenditure of milt. The eggs sink to the bottom and adhere to stones and seaweeds at or near the bottom by means of the sticky egg

envelope, which probably also protects the eggs from injury of various kinds. In correlation with the fact that eggs are fertilized immediately on their emission from the oviduct, it is noteworthy that the spermatozoa live only a few minutes, at most five, in seawater. Consequently, any females that have been isolated for any appreciable length of time could not possibly carry sperm upon their bodies. Therefore any criticism of these results, based on the assumption that occasional heterogenic hybrids that go through successfully to hatching might be due to chance fertilizations by sperm of the same species, have no foundation in fact. All that one has to do to obviate the possibility of any such contingency is to isolate the Fundulus females and handle no Fundulus males during the hybrid experiments. This was done in every case.

The mackerel, in contrast with Fundulus, is a rather large fish, frequenting the off-shore waters except when they come in closer to breed. They are intolerant of water impurities, die soon in aquaria and show themselves extremely sensitive to lack of oxygen or high CO_2 concentration in the seawater. The eggs are about 1 mm. in diameter, typically pelagic in character, but differ from most pelagic eggs in having a distinct pinkish cast. The milt is extremely abundant and is evidently shed in clouds in order to ensure fertilization of the scattered eggs. The spermatozoa live in aquarium seawater for about twenty minutes and probably live even longer in natural seawater.

Differences between embryos and larvae of the Fundulus and mackerel

The egg of the Fundulus develops much more slowly than does that of the mackerel, taking about two weeks to hatch as compared with about two and a half days for the latter. Only in late larval stages are there significant differences of bodily structure and proportions. These do not need description here as none of the conclusions here brought out have to do with such general characters. Our attention must be focused upon one type of character, the peculiarities of the yolk and body chromatophores.

Fundulus has two well-defined types of chromatophore: a type of melanophore (black chromatophore) characterized by large squarish body and few short branches, showing also a tendency to fuse into syncytia, and a red or red-brown chromatophore which in its definitive or fully expanded condition is very intricately branched. The black type, if unhealthy, may remain small, and give out a few slender branches, but is never a very intricately branched cell in pure Fundulus embryos. The red chromatophores also may be relatively simple or unbranched if the embryo is pathological or retarded.

The mackerel larvae also have two types of chromatophore equally characteristic of the species and quite distinct from those of Fundulus. One type is a melanophore which is characterized by a small core or body and very slender anastomosing branches. They never exhibit the tendency to fuse into syncytia. The second type is an olive-green chromatophore that occurs in the larvae both on body and yolk sac. Usually there are two large green cells just back of the eyes, two more a short distance behind the otic vesicles, and two or three adjoining the Kupfer's vesicle on the yolk sac. There are no red cells in mackerel nor any green cells in Fundulus. So in these two opposed characters there is a sharp contrast, and the finding of a red chromatophore in a mackerel egg-hybrid or a green chromatophore in a Fundulus egg-hybrid could not be interpreted as within the range of variability of the maternal species or as a pathological occurrence. A fair-minded critic will admit that the only reasonable explanation is that the factors for the foreign type of cell have been introduced by the foreign sperm.

Methods and general results of the hybridization experiments

During the latter half of June, when both Fundulus and mackerel are at the height of the breeding season, is the best time to make the crosses. Results of experiments made later in the season, although to all appearances development is more frequently normal, may be quite different.

The mackerel egg gives very poor results when inseminated with foreign sperm of any of the numerous species tried. A very large percentage of eggs develop, but many cleave quite irregularly. A somewhat smaller percentage proceed normally or nearly so up to gastrulation and then die. I have never succeeded in raising mackerel eggs inseminated with the sperm of any foreign species up to the point of embryonic differentiation, and hence no chromatophores are formed. This study of heredity is therefore confined to one of the two reciprocal crosses: that produced from the Fundulus egg inseminated with mackerel sperm. The striking difference between the developmental success of the reciprocal crosses is not at all an uncommon phenomenon. I described in a former paper numerous other cases of the same sort (Newman, '15). The explanation seems to be that the mackerel egg is extremely sensitive to foreign sperm material and fails to tolerate it beyond the cleavage period.

A detailed account of a single typical experiment in heterogenic hybridization will serve to bring out the essential facts.

A typical hybridization experiment between Fundulus ♀ × mackerel ♂

The following account gives the details of five experiments performed on June 12 at the Woods Hole fish traps. An unusually fine lot of female Fundulus were segregated from males and taken in a large aquarium in order to have the best possible conditions. A large female was chosen, the eggs stripped into a bacteria dish and inseminated with milt from a vigorous male mackerel just taken from the traps. After about ten minutes the excess of milt was washed out carefully to avoid contamination of the water. Four other exactly similar experiments were performed using other males and females. This one experiment here described gave a somewhat wider range of types than the others, but all gave substantially the same results as far as fundamental matters are concerned. After about five hours the eggs in all experiments were examined and all unfertilized eggs removed. In the five experiments the following percentages of

eggs developed at least as far as the early cleavage stages: 82, 91, 77, 85, 72. There are always a few immature and possibly some overripe eggs and one gets about these same percentages in a pure-bred lots of Fundulus eggs, so that we may conclude that the percentage of fertility of Fundulus eggs with mackerel sperm is about normal.

Although the eggs in these experiments were well cared for and casually examined from time to time, no detailed study was made until nearly one week after fertilization, when the chromattophores had made their appearance in abundance. On the seventh day, however, a complete census of the hybrid embryos was made on the basis of their relative success in development and the heredity of maternal and paternal types of chromatophores. It was possible roughly to divide the entire lot into eight classes, beginning with those that showed the most nearly normal development and ending with those that had died since development began. A full account of the conditions seen on the seventh day is herewith given and is to be compared with another census taken after nearly three weeks.

Class A. A small group of three individuals apparently normal in every way. The heart-beat is strong, the circulation abundant and vigorous. Although a little belated as to stage of development as compared with pure-bred F. heteroclitus embryos of equivalent age, they are exactly like the latter even in the chromatophores which are pure maternal. It may be said that from one to five such embryos appeared in all five experiments and cannot be due to accidental insemination with F. heteroclitus sperm.

Class B. Five individuals are slightly retarded as compared with class A, due evidently to their failure to establish a circulation. The heart in each is large and pulsating vigorously, and in two individuals contains some blood which moves back and forth in the heart chambers as the pulsation drives it. The chromatophores are all of the maternal type except that the black chromatophores are somewhat more branching than one expects in pure-bred Fundulus embryos.

Class C.   In a sense this class is ahead of class B, but in another ranks below it.   Two individuals of a decided paleness and more slender of body than those of class B have established a circulation.   The heart, however, is of small proportions and the red blood corpuscles are few in number.   The blood stream is quite sluggish as compared with that in class A.   The feature of most significance in these embryos that marks them off sharply from those of earlier classes is that they both exhibit paternal heredity, in that green chromatophores are found scatteringly on the yolk, but none on the body.   The black and red chromatophores of the maternal species greatly predominate.

Class D.   Eight individuals showing chiefly subnormal conditions in the head region.   The eyes are small or asymmetrical in size or position; in one the eyes are very close together approximating a cyclopic condition.   The hearts are typical string-hearts pulsating vigorously.   Both Fundulus and mackerel types of black chromatophores are present in abundance, but the maternal type predominates slightly.   Red Fundulus chromatophores are more abundant than green mackerel chromatophores, but the latter are present in abundance.

Class E.   A large group of forty-two individuals, all decidedly subnormal in development, especially with regard to the most anterior structures.   The eyes are of various subnormal types: microphthalmic, monophthalmic, cyclopic, or rudimentary.   The heart is at best a pulsating string-heart, but in many cases no pulsating tissues are noticeable.   The posterior parts of the body are in all fairly well grown.   It is in this group that one finds the most complex biparental combinations of chromatophores. Both the Fundulus square and mackerel branched black chromatophores are numerous, and both Fundulus red and mackerel green chromatophores occur in abundance.   Figures 2 and 3 represent two specimens that are quite representative of this class, drawn a few days after this first census was taken.   One specimen (fig. 2) has about the average condition for the class; the reduced, somewhat vaguely defined head and eyes, the almost equal balance between the maternal and paternal chromatophore types being quite characteristic.   The other specimen (fig. 3) a

somewhat more abnormal head and eyes and a predominance of the paternal tendency in chromatophores, mackerel greens being much more numerous than Fundulus reds.

Class F. A medium-sized group of eighteen individuals that are decidedly more subnormal, especially in apical parts, than class E. These individually show no differentiation of eyes, no otic vesicles, and no heart. The posterior parts of the body vary from quite long slender posterior prolongations to shorter and broader processes. In all of these the mackerel types of chromatophores are at least as prominent as the Fundulus types. In many cases the former greatly predominate over the latter and in four individuals one has to make a careful search to find any chromatophores that are definitely of the maternal type. These last individuals are as pure paternal as some of the hybrid embryos that Loeb described as pure maternal.

Class G. A group of sixteen individuals that are little more than amorphous masses of living cells. Only the transparency of the masses and the expanded condition of the chromatophores show that these amorphous embryos are still living. While the majority of these show distinct biparental inheritance of chromatophore types, two are absolutely pure paternal.

Class H. Twelve embryos that had begun cleavage have died and have been removed from time to time. In no case had they developed beyond early gastrulation.

Excluding the dead embryos, there are seven groups that usually overlap somewhat so that a continuous series might readily be made. The arbitrary classification, however, is a necessary part of the experimental method, for each class was put into a separate vessel and followed throughout the life of the embryos. For about a week after the segregation into classes was made only occasional studies of selected individuals were undertaken and a good many drawings were made for record. On July 1, however, nineteen days after fertilization, the seven classes were subjected to a second complete census with the following findings:

Class A. One individual had hatched on the previous day after eighteen days' development; four or five days late as com-

pared with pure F. heteroclitus. A second individual was drawn and shown in figure 1. This individual is nearly normal, but has not grown to more than half of the size of the hatched individual. The large mass of yolk still remaining is probably destined to remain undigested. This individual lived for over a week longer and never hatched. The third and last individual is very much like figure 1, but somewhat smaller and less advanced. All three show only Fundulus chromatophores, though the black chromatophores are somewhat branched, a condition seen however in many pure-bred Fundulus larvae in stages just prior to hatching.

Class B. All five embryos are still alive, but are decidedly abnormal. The anterior parts are better developed than the posterior parts. They have rather large eyes and the tail fin has failed to differentiate. Although these embryos had never established a circulation, they have evidently been able to assimilate a considerable amount of yolk as is indicated by the diminished mass of the latter. The early observations showed no distinct evidences of paternal heredity and there are still no green chromatophores; so one may consider this group as pure maternal.

Class C. The two embryos in this class now show no circulation. The rather weak partial circulation seen earlier has been inadequate. The development is now decidedly subnormal, the eyes being vaguely defined and the heads small. In both embryos the green chromatophores are much more numerous than when previously examined. Evidently the paternal influence was somewhat belated, but operated strongly to retard development when once it gained momentum. Perhaps this is a case of delayed dominance.

Class D. All eight individuals are still alive, and it is in this group that now occur the best cases of reduced head parts and comparatively well-developed trunk. One individual has an amorphous head and a fairly well-developed caudal fin. One individual is cyclopic with the eye bilobed and directed toward the anterior. The others have narrow heads with small indistinct eyes. The maternal types of chromatophores are still somewhat in the ascendency, but the difference is not so striking as at the former census.

Class E. Forty of the forty-two individuals are still alive. The two dead ones have evidently succumbed to infection. Six others are evidently dying as indicated by cytolylic action and vacuolization of the yolk near the embryos. The remaining embryos belong to two distinct types. Thirty-four show all kinds of eye and other head abnormalities, such as cyclopia, protruding lenses, asymmetrical development of eyes, and rudimentary eyes; in these the rest of the body is fairly well developed, but never differentiated to the point of developing fins. Six individuals show comparatively well-developed heads and eyes, but no trunk differentiation, the body being a minute turned-up stump sticking out from the head. The first lot show much more predominance of the mackerel type of chromatophores than the latter. It seems probable that the mackerel influence has been secondarily suppressed to a large extent in the six individuals with the well-developed heads and reduced bodies.

Class F. All but three of the eighteen individuals of this class have died and have been removed from time to time, leaving only three that show signs of life. These three individuals are, however, of great interest. One has a pulsating membrane near a flat mass of undifferentiated tissue. This rhythmically contracting drum of tissue I interpret as an isolated heart. The other two embryos are examples of isolated eyes. One is certainly an isolated eye, for it has both a pigmented retina and a small lens. The other has a protruding rounded translucent body that I am certain is a lens lying in a flattened cup of tissue which is a very much spread out optic cup. Curiously enough no green chromatophores are to be seen on any of these three embryonic rudiments, although there were undoubtedly some at an earlier period.

Class G. All individuals now dead and disintegrating.

All of the individuals in this experiment were preserved for microscopic study and will furnish material for a histological analysis of the hybrid situation.

No less than seven other experiments performed under conditions essentially identical to those employed in this case gave results much like those recorded. In only one other experiment

did there occur isolated eyes and in no other one was there an isolated heart. In none of the other experiments were there quite as numerous green chromatophores, but in two experiments the green pigment approached that described for frequency and brilliance. In three experiments there was a total lack of types in which the head is well developed and the trunk a mere stump, but in four other experiments one or more typical individuals of this sort occurred. In two experiments there was a lack of cyclopeans, but there were numerous individuals with all of the other types of ophthalmic defects. I have no explanation to offer for these minor differences in experimental results. They may have been due to differences in relative vigor of the parent individuals used in the various cross fertilizations.

DISCUSSION

Chromatophore characters as criteria of heredity in Teleost hybrids

The value of chromatophore characters in the study of hybrid heredity in Teleosts has been abundantly demonstrated in previous papers (Newman, '08, '14, '15; Bancroft, '12). Especially well known are the modes of inheritance of the chromatophores of Fundulus heteroclitus, the square black type and the red type. The branching black chromatophores and the green chromatophores of the mackerel are not so well known, but are equally characteristic.

The inheritance of these four different types of chromatophores deserves detailed attention. It seems evident that the black chromatophores in the two species are homologous structures. The differences between the two types is largely a matter of branching habit and size of cell. In Fundulus heteroclitus the cells in their definitive condition are polygonal, almost without branches and frequently fused into syncytia; in the mackerel the body of the cell is insignificant in comparison with the branches, which form an elaborate reticulum, each cell distinct from its neighbors. In hybrids every admixture of the opposed characters may be found sometimes in a single cell, or adjacent cells may show complete segregation of maternal and paternal

characters.   The different characters of these cells behave as
though they were Mendelian unit characters that are shuffled
and dealt out in all possible combinations.   Some cells show
the maternal character of squarish body plus the fine branches
characteristic of the paternal species.   Branching cells in hy-
brids also fuse into syncytia and nonbranching cells remain
isolated.   A field of chromatophores (fig. 4) is essentially a mosaic
of paternal and maternal characters, and to explain the situation
one must call upon the somewhat overworked idea of somatic
segregation, or else make use of the hypothesis of regional alter-
native dominance, which is, in last analysis, dependent on somatic
segregation.

The red chromatophores of Fundulus and the green ones of the
mackerel are not homologous structures and are in no sense alle-
lomorphs of each other.   One never finds any mixing of the char-
acters of these two types of cell.   They are absolutely distinct
and opposite, and each is quite characteristic of the species to
which it belongs, for Fundulus never has any green chromato-
phores nor the mackerel any red ones.   It will be obvious, then,
that we have the ideal situation for demonstrating paternal
inheritance.   If we find in a hybrid between these two species
the characteristic chromatophores of the paternal species, the
controversy as to the pure maternal character of heterogenic
hybrids is at an end.   The presence of abundant green chromato-
phores in a hybrid based upon a Fundulus egg proves that the
hybrid inherits this character from the paternal species, the
mackerel.   This is not a doubtful matter or a sporadic occurrence,
but a result that anyone can obtain any time he crosses Fundulus
heteroclitus ♀ × Scomber scombrus ♂ toward the middle of
June.   If the cross is made two weeks later it will be difficult to
find the green chromatophores.

*The correlation between success in development and the strength
of paternal inheritance*

It has been pointed out that in this cross, as well as in a con-
siderable number of other heterogenic crosses, that the most

nearly normal hybrids, those that reach an advanced stage of development and occasionally hatch, show the least evidences of paternal heredity—sometimes being pure maternal in their chromatophores and in body form.   If these larvae are absolutely pure maternal, and I see no reason for doubting it at present, the explanation of this hybrid phenomenon offers a very pretty problem in genetics.

Loeb, as we have seen, classes all types of heterogenic Teleost hybrids with his extreme interclass echinoderm 'crosses,' and concludes that they are to be explained as products of a sort of parthenogenesis, the foreign sperm playing merely a development-initiating rôle and not functioning in later development and heredity, except negatively as a retarding agent.

The present studies show that this view is quite unacceptable on three counts:

1. The experiments just recounted prove that the sperm functions in development and heredity.   The same is no less true for other crosses.

2. These nearly normal pure maternal hybrids are merely part of a graded series of types, the next grade showing a slight degree of abnormality and a less positive pure maternal character, and the next grade showing less normal development and positive evidence of paternal heredity.   Other grades show progressively stronger paternal heredity and progressively less normal development.   The question would be pertinent, then: if the most successful and apparently pure maternal hybrids are parthenogenetic, what is the explanation of the rest of the series?   There are no sharp lines of demarkation between the normal and abnormal nor between the pure maternal and the mixed type of hybrid. If one results through the real coöperation of the sperm cell in development, so do the others.

3. The traditional factual basis for the belief in the parthenogenetic nature of heterogenic hybrids is derived from the cytological studies of cross-fertilized eggs of echinoderms.   In those cases the usual situation in very wide crosses is one in which the sperm head enters the egg but takes no part in cleavage, remaining an inert lump in the egg cytoplasm.   In Teleost hybrids the

situation is entirely different. All cytological studies of Teleost hybrids of suborder width show that the paternal chromosomes retain their specific size, shape, and number, and are distributed to the blastomeres nearly or quite normally during the cleavage period. That occasional irregularities in chromosomal distribution occur is seen in this and other types of hybrid in which there occur numerous eggs in which pronouncedly irregular cleavage occurs. Such embryos die in early stages of cleavage or before embryonic differentiation begins. It seems certain, therfore, that in all of those embryos that reach an advanced embryonic stage the chromosome distribution (both maternal and paternal) has been at least fairly normal. Moenkhaus found that the paternal chromosomes were still normal in their distribution in advanced embryonic stages in the Fundulus $\times$ menidia cross, a cross of the same width as that between Fundulus and the mackerel. If, therefore, the paternal heredity materials coöperate during the developmental process with the maternal, it seems logical to expect them to function in heredity. It is hardly conceivable that the chromosomes, which are living entities, should be growing, dividing, and being distributed from cell to cell in cleavage and later development, unless they are functioning, and if they are functioning, how else can they function than in the expression of the changes we speak of as differentiation and heredity?

In view of these considerations, we must seek some other explanation of the pure maternal appearance of the most successful Teleost hybrids. It is my belief that the occasional occurrence of a hatching larva in such a cross as that between Fundulus and mackerel is to be interpreted as a case of early and complete recovery from an initial inhibiting agent. Though the foreign sperm fertilizes the egg and coöperates with it in development and heredity, it must also introduce some materials that are inimical to normal development. This would seem only likely from what we know of the high degree of specificity of protoplasmic materials. In a few cases the egg materials are able either to transform, absorb, or in some other way to neutralize these foreign elements so that they have little or no harmful

effect upon the development.   At the same time the specificity
of the paternal chromatin seems to be neutralized and it is
unable to cope with the unaltered maternal hereditary factors
in the determination of chromatophore and other characters.
Other hybrid eggs react a little less promptly or less completely
to the foreign materials and are in consequence affected by them
more or less strongly.   Any agent inimical to development seems
to express itself primarily as a retarding agent.   As Child has
shown, an inhibiting or retarding agent has the most pronounced
effects upon the structures that have the highest rate of metabo-
lism, usually the head, heart, and especially the eyes.   Conse-
quently, when the foreign materials are most active, as shown by
the presence of many of the paternal type of chromatophores,
the effects of retardation in development are most strikingly
shown in pronounced abnormalities or early death and disinte-
gration of embryos.   In the average case, however, the inhibiting
effect, though long continued, is not unduly severe, and there
result large numbers of embryos with abnormal head parts of all
grades of severity and fairly well-developed posterior structures.
This type of result would be classed as the effects of differential
inhibition.

There occur, however, not infrequently embryos in which the
head parts are better developed than are the posterior parts, or,
in extreme cases, only isolated eyes or hearts occur on an other-
wise undifferentiated blastoderm.   These are, I believe, to be
interpreted as the result of differential inhibition followed by
differential recovery.   According to Child's theories, the apical or
head parts, although most susceptible to retarding conditions,
also have the greatest capacity for recovery when the inhibiting
agent is removed or reduced in intensity.   Evidently some
embryos recover from the inimical effects of the foreign sperm,
either by becoming acclimated or because the foreign element has
grown weak, or perhaps been absorbed, and the anterior struc-
tures take up the process of differentiation and develop principally
eyes and adjacent head parts, the rest of the body remaining
in the original retarded condition incapable of recovery.   Thus
we can account for trunkless heads and even isolated eyes and

hearts. To a certain extent the occurrence of the paternal types of chromatophores bears out this interpretation, for in these recovery cases there are few paternal chromatophores and sometimes none. When the hindering effect is lost, the hereditary effect is correspondingly lost or diminished. Although the parallelism between the occurrence in these hybrids of degree of abnormality and abundance of green (paternal type) chromatophores is not exact, it is certain that lack of green chromatophores is accompanied by nearly normal development and that whenever the green chromatophores occur in abundance development is decidedly abnormal.

The need of better criteria of hybridization

What is a hybrid and by what criteria may a true hybrid be distinguished from a pseudohybrid? These questions have forced themselves upon my attention during the present investigations and demand an answer. Is it scientifically a valid procedure to include as hybrids those egg and sperm unions between representatives of different phyla, such as those between echinoderms and molluscs, and those between different classes, such as Echinoidea and Asteroidea? It is admitted by those who have studied this type of union that the sperm chromatin acts as an inert body within the egg and in no way, except as it hinders development, plays a rôle in development and heredity. Moreover, there is no initial attraction or reaction between egg and sperm in these cases, the union being brought about by the aid of chemical agents that serve to break down specific incompatibility between these foreign materials. I would therefore suggest that these and all similar forced unions between diverse germinal products be called what they are, merely mechanical mixtures of foreign protoplasms. I would further suggest as a definition of hybridization, a natural union between diverse germ cells that involves a fertilization reaction between egg and sperm and in which the sperm chromatin shows evidences of coöperation in cleavage and subsequent development. The acceptance of these distinctions would appear to clear the atmosphere of mis-

understandings. We shall all then be able to admit that these 'mechanical mixtures,' which some authors have called 'heterogeneous' or heterogenic hybrids, are pure maternal and may be parthenogenetic as to the initiation of development. On the other hand, we must expect to find in true hybrids (i.e., all those in which the male germ cell shows morphological evidence of playing a rôle in development) an accompanying physiological effect upon development and heredity. Personally I feel that a morphological picture must represent an equivalent result of functioning and must presage further functioning. It may well be that the egg develops more normally when the activities of the foreign sperm are suppressed, as is evidenced by nearly normal and pure maternal larvae among Teleost hybrids, and that marked activity on the part of the sperm material greatly retards and inhibits normal development; but this is only what we should expect in true hybrid combinations of considerable heterogeneity. In crosses between closely related species, however, the effect of only slightly diverse materials appears to act as a stimulus, and more rapid development and more vigorous offspring frequently result. It is interesting to know that there is every degree of gradation between the two extreme results in true hybridizations. In some extreme heterogenic crosses the functioning of the sperm is so vigorous as to retard normal development during cleavage so seriously that differentiation is wholly inhibited. At the other extreme are those cases in which even during cleavage the processes of development are accelerated and supernormal individuals result. All of these results are rightly to be included within the category of hybridization phenomena.

SUMMARY

1. A survey of the literature on Echinoid hybridization is attempted in order to gain an accurate idea of the relative availability of Echinoids and Teleosts for the study of hybridization phenomena. It appears that by chemical means inseminations of Echinoid eggs with sperm of other orders, classes, and even phyla may be made, but these involve no real fertilization reac-

tions and are considered as mechanical mixtures. Real hybrid phenomena appear to be restricted to species within the order Diademoida of the class Echinoidea.

2. In fish hybridization the same situation appears. The hybrids known for fish are restricted within the confines of the order Teleostei.

3. Subordinal crosses in both Echinoids and Teleosts have been made, but the data for crosses of this width among Echinoids is contradictory and very unsatisfactory; hence the especial value of an intensive study of Teleost hybrids of this width of cross. Practically all Teleosts may be crossed without artificial chemical aids.

4. As an example of a heterogenic Teleost hybridization of suborder width, that between Fundulus heteroclitus and Scomber scombrus is chosen after an extensive survey of a wide range of crosses, because it is especially well adapted to show the facts about heterogenic hybridization in a clean-cut and unequivocal way. Other crosses nearly as good might have been chosen.

5. Although the adult characters of the two species are in striking contrast and the larvae at all stages are different in all details, the only reliable differentiating characters of the younger larval stages are the chromatophores. Fundulus has red chromatophores and the mackerel green ones; Fundulus has solid black chromatophores and the mackerel delicately branched black ones; these differentiating characters serve to indicate the degree to which the two parental elements function in development and heredity.

6. Cross fertilizations between the two species result differently according to which is used for the egg species. When Fundulus eggs are used, all grades of success in development up to hatched larvae are obtained. When mackerel eggs are used, all embryos die before or during gastrulation, before any specific characters are differentiated. The study of heredity is therefore confined to one of the two reciprocal crosses, that between Fundulus ♀ and Scomber ♂.

7. If the experiment is performed near the middle of June, when the breeding season of both species is at its height, one gets

results nearly uniformly as indicated in the typical experiment given in detail. Later in the season the results differ materially. In this paper our attention is confined to the results obtained in a number of crosses made about June 12 to 15.

8. The general conclusion derived from a study of the data are: in proportion as the paternal element vigorously exercises its functions, in like proportion is development retarded and the various types of monster appear. Likewise the paternal types of chromatophore tend to be more numerous the more pronounced the general abnormality. The most successful embryos are, in so far as the chromatophores are concerned, pure maternal, a fact that leads to the belief that these practically normal individuals are not the product of parthenogenesis, but represent cases of complete recovery from an influence that in most cases retards development. It seems that the disharmonious elements of the paternal contribution are either completely neutralized or so modified as not to oppose the normal activities of the maternal materials.

9. The great majority of hybrids are subnormal, especially in their apical structures (eyes, hearts, etc.), and at the same time show obvious paternal heredity. The persistence of paternal types of chromatophores indicates that the paternal contribution is still active and that complete recovery is therefore impossible. The embryos are largely to be classed as the results of permanent but nonlethal inhibiting agents, the effect of which is always, according to Child's 'axial gradient' hypothesis, to produce embryos with more abnormalities in the apical than in the basal parts. Hence we explain the wide range of ophthalmic and cardiac abnormalities in these hybrid groups as the result of differential inhibition.

10. A somewhat smaller group of hybrid embryos show large apical parts and reduced basal parts. Occasionally isolated apical parts (eyes and hearts) occur in an otherwise undifferentiated egg. Such embryos are usually pure maternal as to chromatophores or at least in the region of differentiation. Such anomalies are to be interpreted as differential recovery products, but differ from the complete recovery cases in that recovery

occurs only after a prolonged inhibition. When too long inhibited the whole organism cannot completely recover, but only the apical structures, whose capacity for recovery has been shown by Child to be greater than in basal structures. Thus we find heads differentiated without bodies, or with at best rudimentary bodies, and occasionally isolated eyes and hearts.

11. These experiments may be taken to indicate that in heterogenic crosses no harmonious structural differentiation can result, without the neutralization or elimination of the disharmonious paternal materials. If the latter function actively, so as to express this functioning in heredity, there can result only retarded and subnormal embryos and larvae, and the vast majority of heterogenic hybrids are of this type.

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PLATE 1

1. A hybrid embryo (Fundulus ♀ Scomber ♂) nearly three weeks old. It is nearly normal in structure and practically pure maternal in the chromatophore complex. Note the total absence of the green chromatophores of the paternal parent species, as contrasted with their presence in figures 2, 3, and 4. The egg is 2.5 mm. in diameter.

2 A subnormal hybrid embryo (Fundulus♀ Scomber♂), showing the anterior end of the body and the rather poorly differentiated eyes. On the large yolk mass note both the square black chromatophores of Fundulus and the branching black chromatophores of Scomber. Note also that the red chromatophores of Fundulus and the green ones of Scomber are about equally numerous. The drawing was made about two weeks after fertilization.

(Drawn from life by Kenji Toda.)

1

2
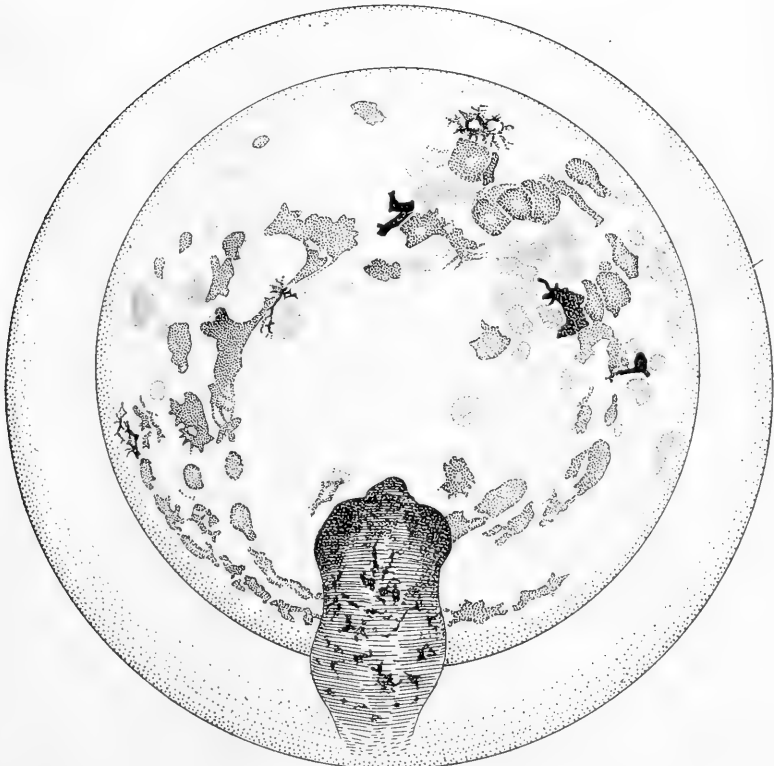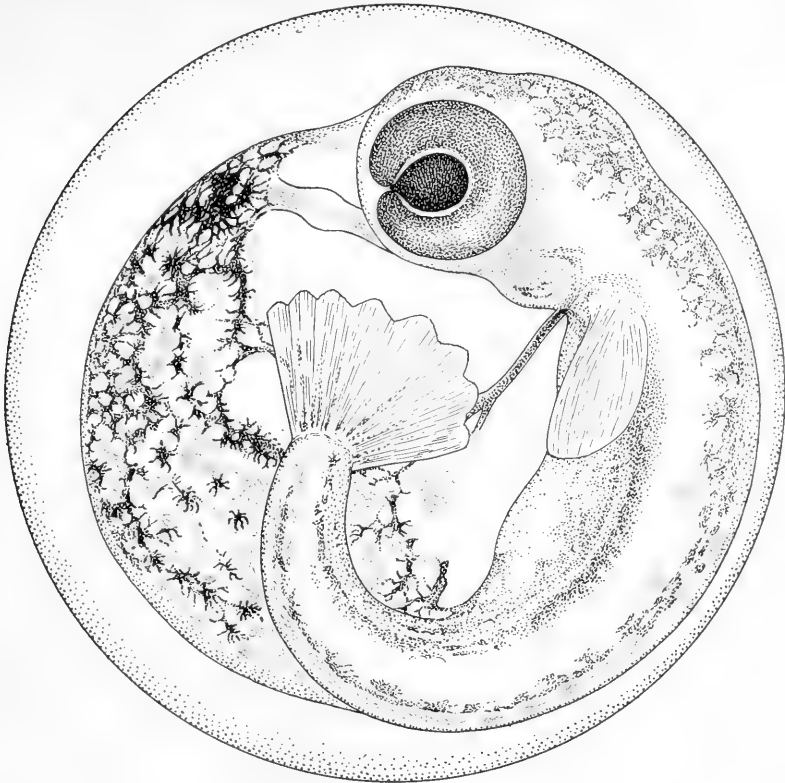
3   A subnormal hybrid embryo (Fundulus ♀ Scomber♂), two weeks old, show-ing abnormal eyes and a rather pronounced ascendency of the paternal types of chromatophores.   Only a few of the red chromatophores of Fundulus occur, while the green chromatophores of the Mackerel are numerous.   The whole embryo has a greenish cast that one never sees in a pure-bred Fundulus embryo.

4   A sample field on the yolk-sac of a typical hybrid embryo (Fundulus ♀ Scomber ♂), two weeks old, showing all four types of chromatophores: the solid squarish blacks of Fundulus (they were not particularly square in this field), the finely branching blacks of Scomber, the reds of Fundulus and the greens of Scomber.   The field drawn was not chosen to emphasize any point, but was selected by the artist with scarcely any direction.
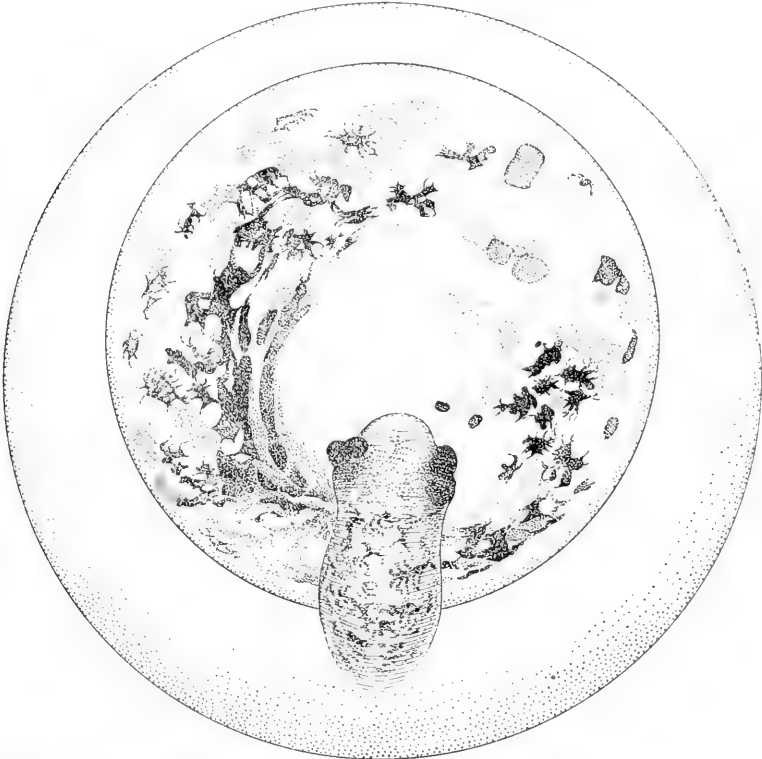
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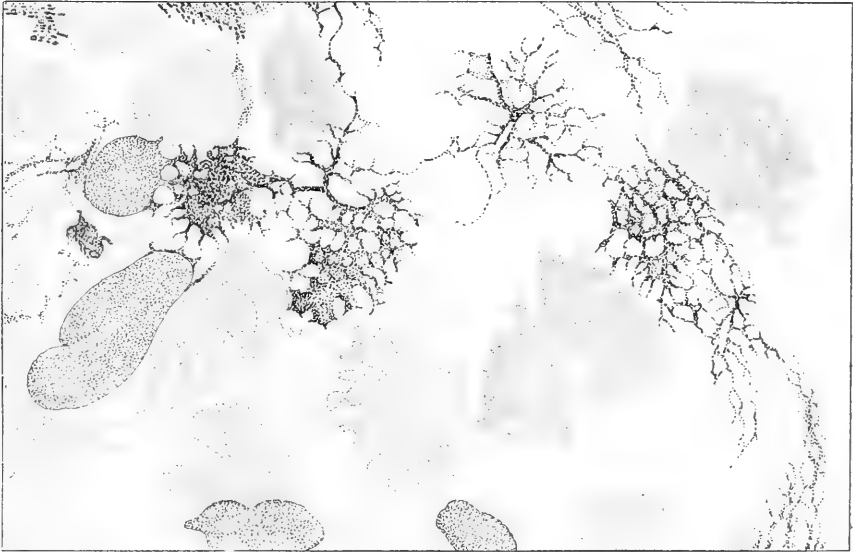
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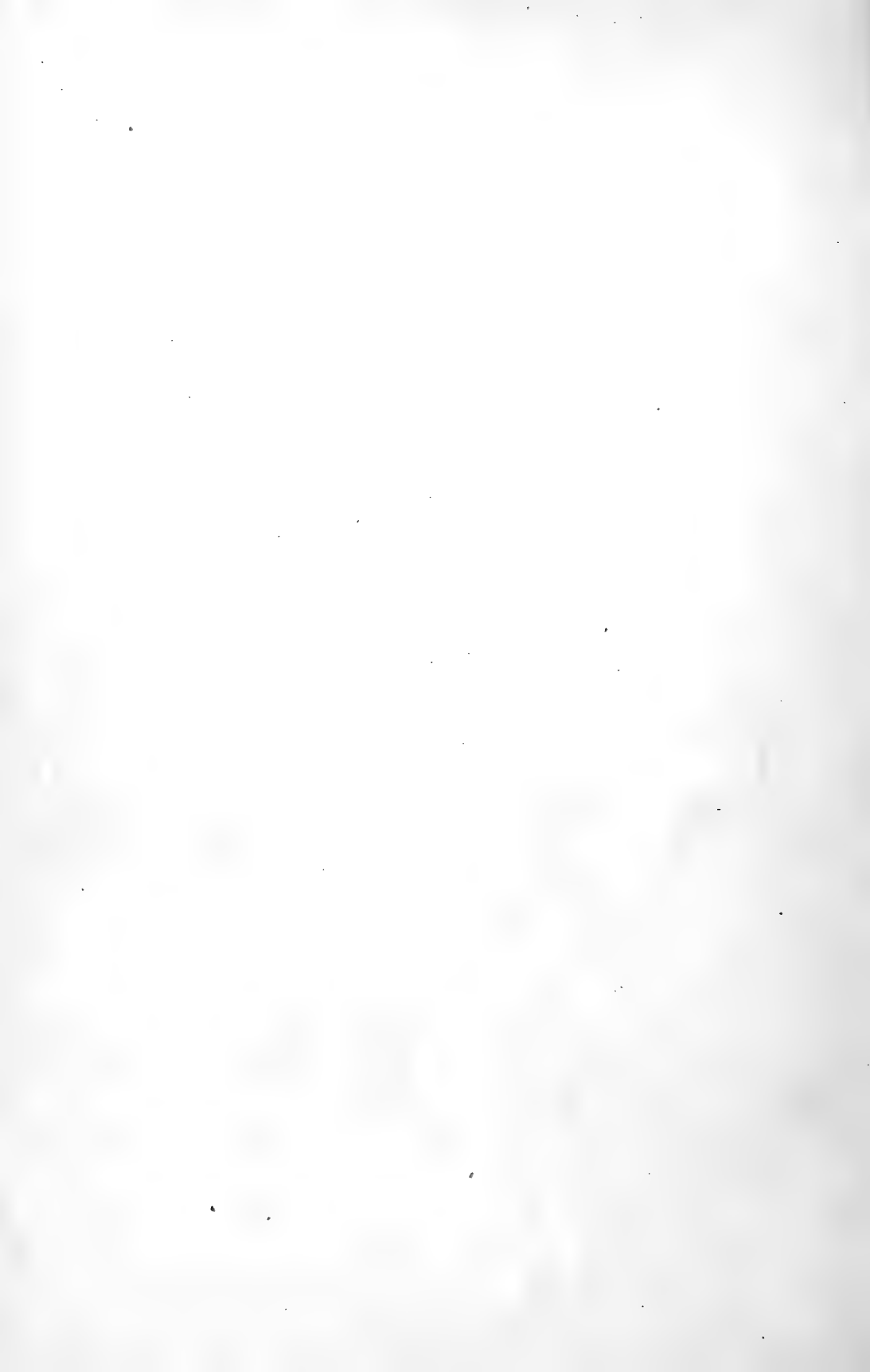
LIGHT REACTIONS AND METABOLISM IN MAY-FLY NYMPHS

I. REVERSALS OF PHOTOTAXIS AND THE RESISTANCE TO POTASSIUM CYANIDE

II. REVERSALS OF PHOTOTAXIS AND CARBON DIOXIDE PRODUCTION

W. C. ALLEE AND E. R. STEIN, JR.

Lake Forest College

FOUR CHARTS

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I. REVERSALS OF PHOTOTAXIS AND THE RESISTANCE TO POTASSIUM
CYANIDE[1]

HISTORICAL

The idea that there is a relation between the fundamental metabolic processes and the signs of the reaction of animals to stimuli is not new. It has also occurred to some investigators that there may be a relation between the rate of these metabolic processes or part of them and the phototactic reaction. Holmes ('05) found that Ranatra is made more positive by conditions that cause an increase in activities and made more negative by opposite conditions. Carpenter ('05), studying the light reactions of Drosophila, concluded that the more stimulated the animals were, the more positive they became.

Jackson ('10) came to the conclusion that the changes in responses to light, which he obtained with the amphipod Hyalella, are not due to chemical changes of the eye or skin, as Loeb ('10) had suggested, but are rather due to a sudden stimulation or shock to the nervous system.

Mast ('11, p. 283) states that in Arenicola, "Any condition which serves as a depressant tends to cause the young larvae to become negative," and he concludes (p. 287) that "The facts 1) that the light reaction may be affected in a given organism by so many contrasting conditions; 2) that the same change in external conditions may cause opposite reactions indifferent organisms, and 3) that the sense of the reaction may be changed without any immediate external change—indicate that these responses are due not to a direct and specific effect of the environment on some definite chemical compound within the organism, but rather to the effect on the organism as a whole."

Bohn ('12) separately reached a somewhat similar conclusion when he decided that there are two kinds of sensibility, one to light and one to shade, and that these correspond to antagonistic

chemical reactions.   Causes that accelerate oxidations tend to make animals positive to light and causes that inhibit oxidations produce reactions to shade.   Bohn's conclusions are based on his own and Drzewina's ('11) observations.

Phipps ('15) found that when amphipods, negative to light, are treated with potassium cyanide, chloretone, or were subjected to decreased oxygen tension or to starvation that many of the animals reversed their reactions both to light intensity and to the direction of rays.

None of these workers measured the effect of the substances upon the metabolism of the animals under investigation.   Indeed, this has been done only by MacCurdy ('13), who found that the negative starfish Asterias forbesi gives off less carbon dioxide in sunlight than in shade.   He made no effort to control the reaction to light.

Many investigators, beginning with Loeb ('04), have recorded reversals of sign of the phototactic response which could be produced at will; the problem which we set for ourselves was to find whether or not there is any correlation between the sign of the reaction to light and the rate of metabolism of the animal as measured by resistance to potassium cyanide or by carbon dioxide production.   For the first part of this inquiry the May-fly nymphs Leptophlebia sp? and Epeorus humeralis (Morgan) were chosen because of their abundance near the laboratory and because Wodsedalek ('11) had found the phototactic reactions of the May-fly nymph Heptagenia interpunctata is readily reversed by chemicals.

ECOLOGICAL NOTES

The Leptophlebia and Epeorus nymphs studied lived in mountain streams with stony beds and rapid currents such as are quite common in the Berkshire Hills.   Their distribution was most carefully studied in Tunnel Brook (near Hoosac Tunnel).   In the early autumn when few leaves had fallen the nymphs lived on the under sides of stone and were almost invariably facing upstream.   Their clinging ability enabled them to maintain them-

selves in the swift current.   In April and May large numbers of
Leptophlebia were found among submerged leaves in the quieter
parts of the brook.   At the same time Epeorus was most abun-
dant under stones in more rapidly moving water.   Neither were
found on the upper surface of stones until late in May when the
adults were emerging in large numbers.   At this time hundreds
of Epeorus nymphs could be seen on the upper side of any large
stone in the brook, all headed against the current.

<center>METHODS</center>

The phototactic tests were made in a large, light-tight wooden
box to which light was admitted through adjustable slits.   The
opening was near the bottom of the box so that the light entered
the end of the experimental dishes.   During the experiments
described in the first part of this report a north window fur-
nished the light source.   The experimental box was painted dead
black inside.   The rear was curtained with a heavy black, cloth.
A semicircular opening in the floor of the box opposite the light
inlet permitted the observer to sit with head and upper body in-
side the box without introducing an appreciable amount of light.

The light-reaction tests were carried on in oblong glass dishes
measuring 12 x 5.2 x 2.2 cm., which were placed with the long
axes parallel with the light rays.   When it was necessary to cool
the dishes to keep the temperature at or below tap temperature,
the experimental dishes were placed in shallow glass trays and
packed in ice on all sides except that toward the window.   Control
dishes were kept under identical conditions save for the factor
under experimentation.

The nymphs were kept in the laboratory in aluminum tea balls
which hung in running tap water which was similar in salt and
gas content to the water of their native streams.   Most of the
experiments were performed before the animals had been in the
laboratory five days.

REVERSALS OF PHOTOTAXIS

In experiments upon phototaxis the nymphs were selected from a number which had stood a short time in tap water exposed to the light conditions under which the test was to be made. Then, if the experiments were to be upon negative animals, only decidedly negative nymphs were selected. During the course of the experiments nymphs were subjected to various strengths of sulphuric, hydrochloric, and acetic acids; potassium and sodium hydroxide; potassium, sodium, calcium and magnesium chlorides; ethyl alcohol, chloretone, caffein, strychnine, and to temperature changes.

The two species with which we worked reacted oppositely to light. Epeorus was normally positive while Leptophlebia was normally negative. The former is the more definite in its reaction, as shown by the ratios of the average negative (N) and positive (P) responses of the controls. Thus Epeorus with seventeen sets of control readings gave a $\frac{P}{N}$ ratio of $\frac{90}{11}$ while Leptophlebia with thirty such controls gave $\frac{5}{12}$.

Quantitative reversals were obtained with Epeorus with ethyl alcohol, calcium chloride, and with a decrease of temperature. Alcohol was the most efficient reversing agent used with this species. At tap temperature (about 12°C.) the best results were obtained with a 2 per cent solution. When the temperature was lowered 5° or more, better results were obtained with a 1 per cent solution. Under both conditions quantitative reversals were obtained with positive nymphs and with the control strongly positive throughout.

With Leptophlebia more of the chemicals tried gave quantitative reversals. Hydrochloric and sulphuric acids, potassium and sodium hydroxides, potassium cyanide, and potassium chloride gave 100 per cent reversals of negative nymphs while the controls were predominantly negative throughout. Sodium and calcium chlorides gave 80 per cent reversals with the controls over 80 per cent negative. Magnesium chloride, chloretone, and caffein had little effect. Alcohol, as with the other species, gave a high percentage of reversals, although quantitative results were rare.

TABLE 1

Showing the results of tests as to whether 0.00001 normal solutions of potassium cyanide directly measures the metabolic rate of Epeorus nymphs and 0.000001 N. indirectly measures it. Also as to whether 0.0025 N. directly measures the rate of metabolism of Leptophlebia. Division A of the table exhibits results on animals that should have theoretically the lower rate of metabolism

STRENGTH OF KCN		Division A							Division B							
	NUMBER TESTED	SIZE LIMITS	AVERAGE SIZE	TEMPERATURE	STIMULATED OR UNSTIMULATED	SURVIVAL TIME	NUMBER TESTED	SIZE LIMITS	AVERAGE SIZE	TEMPERATURE	STIMULATED OR UNSTIMULATED	SURVIVAL TIME	RATIO OF DIFFERENCE TO PROBABLE ERROR	SUPPORT EXPECTATION	STATISTICALLY SIGNIFICANT
		mm.	mm.	deg. C.		minutes		mm.	mm.	dec. C.		minutes			
Epeorus																
0.00001 N	42	6.0–9.0	6.9	As B.	※	153 ± 8	50	3.0–5.5	4.4	See A	※	111 ± 6	3.0	*	*
0.00001 N	17	3.5–8.0	6.0	As B.	※	163 ± 12	19	4.0–8.0	5.7	See A	&	92 ± 4	4.3	*	*
0.00001 N	8	3.5–7.0	5.9	7	※	247 ± 20	84	4.0–8.5	5.6	13	※	148 ± 16	2.8	*	*
0.000001 N	19	6.0–11.0	8.5	As B.	※	48.5 ± 4.3 hrs	10	3.3–5.0	4.6	See A	※	97.7 ± 9.4hrs	3.6	*	*
Leptophlebia																
0.0025 N	4	8.0–8.5	8.3	As B.	※	79 ± 6	57	7.0–8.5	7.8	See A	&	49 ± 0.6	4.5	*	*
0.0025 N	85	6.0–8.5	6.8	As B.	※	231 ± 7	153	5.0–5.5	4.9	See A	※	169 ± 10	3.6	*	*
0.0025 N	49	6.0–11.0	7.6	2–4	※	559 ± 13	85	6.0–8.5	6.8	11	※	231 ± 7	16.4	*	*
0.0025 N	6	7.5–8.5	8.3	0–1	※	366 ± 22	4	8.0–8.5	8.3	17–21	※	79 ± 6	10.0	*	*

※ Indicates that the animals were not mechanically stimulated.

& Indicates that they were mechanically stimulated to actively move.

DOES RESISTANCE TO POTASSIUM CYANIDE MEASURE THE RATE OF METABOLISM IN MAY-FLY NYMPHS?

In this work the cyanide resistance method of Child ('13) was used without modification except in the strength of the cyanide solutions employed. In this method with a relatively high concentration the animals with a higher rate of metabolism (cf. Geppert, '99, Hyman, '16) die before those with a lower rate. In much weaker solutions acclimatization occurs and the effect is reversed. The resistance of the nymphs was tested in 500-cc. Erlenmeyer flasks. Control experiments showed that the more sensitive Epeorus nymphs could live from five to fifteen days in this amount of unchanged tap water, provided the temperature was approximately constant.

Some difficulties were encountered in determining the death point. The nymphs were usually observed every thirty minutes in later experiments every twenty minutes), and those that were apparently motionless were removed with a large pipette and carefully inspected under a lens. If no motion was apparent they were stimulated with a needle. If they failed to show any motion under these conditions they were considered dead. It is obvious that this treatment would stimulate live nymphs and more frequent inspection would increase rather than decrease the experimental error.

Epeorus proved to be much less resistant to the cyanide than Leptophlebia. With the former (table 1) a solution 0.00001 normal gave a measurement of metabolism by the 'direct method;' with the latter the same measurement was obtained by a strength of 0.0025 normal. With Epeorus 0.000001 normal was found to measure metabolism by the acclimatization method. With this dilution so great care was necessary in order to maintain the solution at even its approximate strength that no serious tests were run.

A new solution of 0.1 normal potassium cyanide was made up weekly, and from this dilutions were made to the desired strength. With extreme dilutions and with certain preliminary experiments which ran several days the solutions were made up fresh twice daily.

The inquiry as to whether resistance to potassium cyanide in May-fly nymphs is affected by the rate of metabolism of the animal was prosecuted along the following lines:

1) What is the relation between the resistance of large (old) and small (young) nymphs?

2) What is the effect of stimulation upon the resistance to the cyanide?

3) What is the effect of differences in temperature?

The results of these experiments are summarized in table 1. Nymphs of Epeorus were less resistant to 0.00001 normal potassium cyanide when they were small, or stimulated, or at increased temperature. All of these conditions cause a higher rate of metabolism (Child, '13; Allee, '14). On the other hand, in a 0.000001 normal solution the smaller (younger) nymphs were more resistant than the larger ones, which is what the theory demands if a solution of this strength to measure indirectly the rate of metabolic processes.

Leptophlebia in 0.0025 normal solution was less resistant when young, or stimulated, or when the temperature was increased so that this strength of cyanide directly measures the metabolic rate of these nymphs. A solution 0.00025 did not indirectly measure the rate of metabolic processes of these nymphs and the experiments were not continued long enough to find a solution strength that would do so. In all the above instances in which the evidence is that cyanide resistance does measure the rate of metabolism the differences in the survival times exceeds twice the probable error and we may safely hold them to be statistically significant.

RELATION BETWEEN THE SIGN OF LIGHT REACTION AND RESISTANCE TO THE CYANIDE

1. Epeorus

The average survival time of fifty-one positive Epeorus nymphs (table 2) which had been taken directly from tap water was 108 ± 5 minutes. These nymphs averaged 5.4 mm. long. A reversal of the phototactic reaction of forty-seven other posi-

tive Epeorus nymphs was caused by treatment with alcohol (1 or 2 per cent) and decreased temperature (2° to 8°C.). These reversed nymphs gave a survival time of 131 ± 6 minutes in the same strength of cyanide. Their size average was 6.5 mm. The difference in survival time is only 2.1 times the probable error, and since the difference may have been affected by the difference in size, too much emphasis cannot be laid on these results.

The survival time of eighteen nymphs of the same species that failed to reverse in the alcohol-reduced temperature treatment was 88 ± 3 minutes. Their average length was 6.1 mm. The nymphs that were reversed by this treatment lived forty-three minutes longer in the cyanide than those that remained

TABLE 2

Showing the relation between the sign of the phototactic reaction of Epeorus nymphs and their resistance to potassium cyanide

NUMBER TESTED	AVERAGE LENGTH	AVERAGE SURVIVAL TIME	TREATMENT BEFORE KILLING	NUMBER TESTED	AVERAGE LENGTH	AVERAGE SURVIVAL TIME
Positive nymphs				Negative nymphs		
	mm.	minutes			mm.	minutes
51	5.4	108 ± 5	Tap water			
18	6.1	88 ± 3	Alcohol	47	6.5	131± 6
			Lowered temperature	11	6.1	160±16

positive. Since this is 4.8 times the probable error, it must be significant.

The majority of the Epeorus nymphs treated with alcohol and decreased temperature reversed their reaction to light and had a slightly (perhaps questionably) lower rate of metabolism than the control animals and a decidedly lower rate than those not reversed by the treatment. The nymphs that remained positive, although given the alcohol-low temperature treatment, apparently had been stimulated by the alcohol, for they showed a higher rate of metabolism than the control animals. This can be explained by assuming that the alcohol acted as usual, first stimulating and later depressing. If this be true, the fact that alcohol plus reduced temperature was more effective than the

latter alone becomes significant if the metabolic differences prove to be causal rather than incidental or resultant, for the difference between a stimulated period followed by depression may well be greater than mere depression.

Sometimes quantitative reversals were obtained by reducing the temperature. Eleven nymphs that had been thus reversed (average length 6.1 mm.) were killed in cyanide and gave an average resistance of 160 ± 16 minutes. This is fifty-two minutes longer than that given by the control nymphs which is 2.5 times the probable error and is probably significant.

Taken altogether, the evidence here presented indicates that reversed Epeorus nymphs have a lower rate of metabolic activity than do positive animals. One other observation confirms this idea. Epeorus nymphs collected in October were kept in a large aquarium that also contained some fresh-water mussels. In December and January the nymphs were found to be dying in large numbers. When tested all were negative to light, although when first collected they had given almost quantitatively positive reactions. Obviously the metabolic process of the nymphs was strongly retarded and this is correlated with their reversal to light.

2. Leptophlebia

Leptophlebia nymphs were usually negative in their reaction to light giving a ratio of twelve negative to five positive animals. The average survival time of sixty-one untreated nymphs (average length 7.9 mm.) that gave the usual negative light reaction was 131 minutes. Forty-two positive nymphs (average length 7.8 mm.) under conditions similar in every way resisted the same strength of cyanide 130 minutes. Thus there was no difference in the metabolic condition of these two groups that could be measured by the cyanide resistance method.

Hydrochloric acid was very effective in causing reversals in Leptophlebia. The survival time of thirty-six nymphs so reversed (average length 7.7 mm.) was 127 minutes. Twenty nymphs similarly treated that remained negative (average length 7.8 mm.) gave a mean survival time of 120 minutes. This

difference is of course negligible as is also the difference between these acid-treated animals and the control.

Alcohol was also effective in making these negative nymphs positive. Fourteen nymphs that were made positive gave a resistance of 91 ± 7 minutes to the cyanide as compared with 130 ± 4 minutes for the sixty-one control animals. This is 3.5 times the probable error. The eleven nymphs that were treated with alcohol and remained negative showed some stimulation

TABLE 3

Showing the sign of phototactic reaction of Leptophlebia nymphs and their resistance to cyanide

NUMBER TESTED	AVERAGE LENGTH	AVERAGE SURVIVAL TIME	TREATMENT BEFORE KILLING	NUMBER TESTED	AVERAGE LENGTH	AVERAGE SURVIVAL TIME
Positive nymphs				Negative nymphs		
	mm.	minutes			mm.	minutes
36	7.7	127	HCl	20	7.8	120
14	7.8	91 ± 7	Alcohol 2 per cent	11	7.7	109 ± 9
9	7.8	119	H$_2$SO$_4$			
8	7.8	115	Acetic acid	2	7.0	136
7	8.5	127	NaOH	8	7.8	146
8	6.9	132	KOH	2	7.0	130
9	6.8	133	KCN	2	7.4	103
3	8.3	115	KCl	2	8.0	103
6	8.7	100	NaCl	4	8.7	105
			MgCl	2	8.0	129
2	7.8	129	CaCl$_2$	3	7.8	123
6	7.8	161	Chloretone	8	8.1	165
108	7.8	123	Totals and averages	64	7.8	129
42	7.9	131 ± 4	Tap water control	61	7.8	130 ± 4

when tested with cyanide, but not so much as the positive nymphs.

Considering the numbers tested and the small deviation from the resistance shown by the control animals, none of the results obtained with other reagents and listed in table 3 are significant with the exception of those with chloretone. This drug was not very efficient in causing reversals, but did cause decided depression, as measured by the cyanide method, and caused reversals in about 30 per cent of the nymphs treated.

The average effect of all these reagents upon the resistance to cyanide, if such is worth anything, shows no marked difference between positive and negative treated animals nor between the treated animals and the control.

From these experiments with Leptophlebia we have the interesting results that these nymphs were reversed without affecting their resistance to potassium cyanide (HCl and averaged results); with accompanying stimulation (alcohol) and with accompanying depression (chloretone).

The quantitative reversal of positive Epeorus nymphs and negative Leptophlebia by 1 per cent alcohol in the same experimental dish at the same time was repeatedly demonstrated. Thus conditions absolutely identical caused opposite reversals in the two species. At first sight this would appear to mean that both positive and negative animals were reversed for the same reason. This is not necessarily true. In the tests to find the strength of potassium cyanide that would directly measure the metabolic rate of the two species it was found that Leptophlebia was only one-fourth as sensitive as Epeorus. Since the Leptophlebia are much more resistant, a strength of alcohol that only stimulated them may have clearly depressed Epeorus. That such is the true explanation is indicated by the effect of alcohol on the resistance of the two species of nymphs to cyanide. The Epeorus that had been made negative were found to be depressed, while the Leptophlebia that were made positive were clearly stimulated.

II. REVERSALS OF PHOTOTAXIS AND CARBON DIOXIDE PRODUCTION[2,3]

METHODS

The experiments upon which the second part of this report is based were carried on at Lake Forest College upon a May-fly

[3] The nymph whose reactions are described in the second part of this paper is *Heptagenia pulchella* Walsh. We are indebted to Professor J. G. Needham for this identification.

nymph belonging to the Heptageninae. These nymphs are quite common in Pettibone Creek (Shelford, '13, maps) where they are usually found in the stones between riffles. Pettibone Creek is a brook about the same size as the Berkshire streams mentioned in the preceding part, but with much less rapid current.

The nymphs were kept in the laboratory for long intervals during the winter in well aerated running-water aquaria. Animals to be experimented on were transferred to room-temperature aquaria aerated by means of an air-pressure pump operated by water pressure.

The experiments were conducted as at Williamstown save that a daylight, concentrated filament, 100-watt Mazda (C2 of the General Electrical Company) was used as a source light. The experimental box was placed in a darkened room so that only light from the source lamp could enter during the experiment.

In most of this work an assistant plotted the reactions of individual nymphs to light and manipulated their change to experimental solutions. Careful controls were run. When the nymphs were to be changed to a new experimental solution, the controls were changed in the same manner to fresh tap water. The assistant also prepared the pairs of nymphs for their carbon dioxide test in such a way that the experimenter had no idea which was the experimental and which the control nymph.

The carbon dioxide production was determined in Tashiro's biometer (Tashiro, '13, '17) as follows: The assistant placed two nymphs, whose rate of carbon dioxide production was to be compared, momentarily on filter paper and then transferred each to a shallow glass cell.

The nymphs used were of the same size and were selected so that the experimental factor was the only known cause for variation in their rate of carbon dioxide production. The containers were marked for future identification. These were handed to the experimenter and immediately placed in the apparatus. Within five minutes from their removal from the experimental dishes one could get an indication of their relative rate of carbon dioxide production. Under optimum conditions the entire process of determination could be repeated at the rate of three per hour. This was much in excess of the usual rate.

TABLE 4

9/1/16.   1:20 p.m.   Put two lots of eight May-fly nymphs each in aquarium water in dark box.   Light: 60-watt Mazda, 50 cm. distant.   The nymphs had been in the laboratory two days.   Temperature aquarium 21.5; of room 22.

	LOT 1					LOT 2			
	+				−	+			−
1:30					8	1		2	5
1:32	1	1			6	2	1	5	
1:48	1				7	2			6
1:53		2		1	5			8	
2:01			3		5	1	1		6
2:06		1		2	5		1 ·	2	5
2:07		1	1	1	5		1	1	6
2:08		1	1	1	5			1	7
2:09	1	1			6			1	7
2:10	1	1			6			1	7
2:11		2		1	5			2	6
2:12	1		1		6			2	6
2:13	1		2		5			2	6
2:14		1	1		6			1	7
2:15			2		6			1	7
2:18	1*				7*			1	7

* Positive one has been positive throughout put in biometer 2: 20 with a negative one that has been negative all the time.

Washed with CO₂ free air 5 minutes.
Positive nymph in left chamber, negative in right.
Bubble 2: 25.
First ppt. Rt. 2: 28.
Much more left 2: 38.
Took out biometer, 2: 40.   Both active; put back in dark box in separate dishes.
3: 00   Both reacting as before testing in biometer.
3: 20   Do.
3: 45   Do.
4: 10   Do.
4: 15   Replaced in biometer as before.
4: 25   Bubble.
4: 27   First left, positive.
4: 40   More on left as before.
4: 45   Out of apparatus.   Reintroduced at negative end of separate dishes.
5: 00   Nymph that had been positive throughout, still positive; other negative as before.
6: 00   Both negative.
7: 40   Nymph negative throughout now positive, other negative.   Light on all night.
8: 00 a.m.   Both negative.

Some idea of the nature of experimentation together with its effect on the nymphs may be gained from table 4.   This table is a slightly expanded copy of a portion of the laboratory record for the day.   It shows that during almost four hours of observation, in which time three biometer tests were made, the nymphs maintained their original light reaction and that each test showed the positive nymph had the higher rate of carbon dioxide production.

Whatever the faults of the method used, it at least has the merit of being always comparative.   As was to be expected, large nymphs gave off carbon dioxide more rapidly than small ones and active nymphs more rapidly than inactive ones.   These nymphs are strongly thigmotactic, and this usually caused them to remain quietly in their container.   Occasionally one would move.   Such determinations were of course thrown out.

PHOTOTAXIS AND CARBON DIOXIDE PRODUCTION IN UNTREATED NYMPHS

These nymphs, like Leptophlebia, are usually negative to light. This normal reaction to light is graphically shown in chart 1 together with the preliminary and control records shown in charts 2, 3 and 4.

About 20 per cent of the untreated nymphs were positive to light.   When these were tested they were found to have a higher rate of carbon dioxide production than the negative nymphs. Their $\frac{P}{N}$ ratio, based on 332 non-selected control readings was 5.5 This is indicated by the graphs in columns 1 and 2 of chart 1 and the details are given in table 5.

Exposure to light for considerable time sometimes caused negative nymphs to reverse their light reaction and become positive.   Such animals were found to be more stimulated, as determined by the rate of carbon dioxide production, than similar nymphs that had not been exposed to light (table 6).   This is just the opposite to the result obtained by MacCurdy with negative starfish which he found gave off less carbon dioxide when exposed to strong light.

Chart 1

438
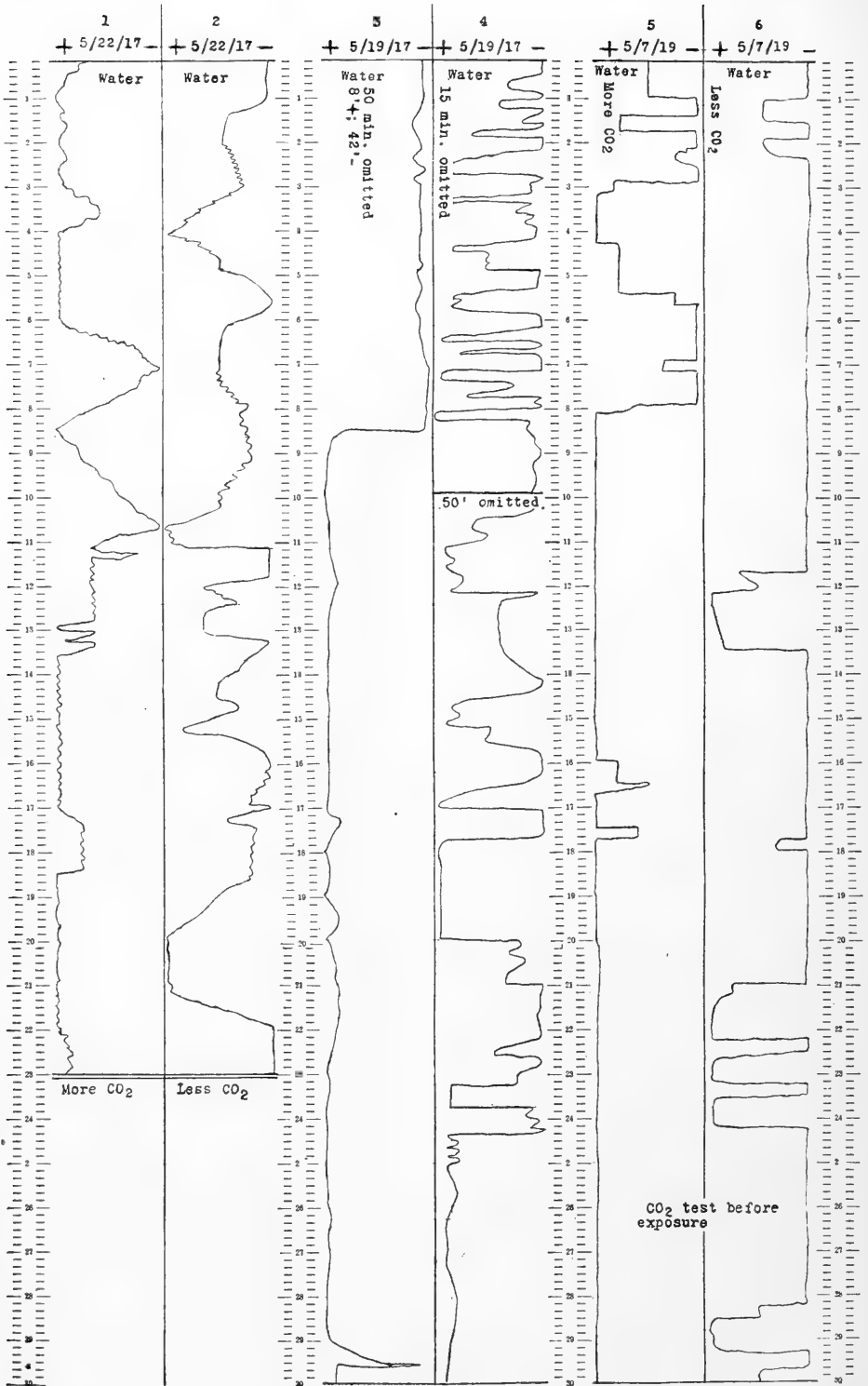
TABLE 5

Showing the comparative rate of carbon dioxide production of positive and negative · nymphs under control conditions (p. 525)

DATE	SIZE		MORE CO_2
	+	−	
	mm.	*mm.*	
9/ 5/16	8.0	8.0	Positive
9/ 1/16	6.0	6.5	Positive
	6.0	6.5	Positive
9/ 2/16	5.5	6.0	Positive
	5.5	5.5	Positive
10/26/16	7.0	7.0	Positive
10/27/16	7.5	8.0	Positive
10/28/17	7.1	7.0	Positive
	7.0	7.0	Negative. More active before test
	8.0	8.0	Negative. More active before test
11/ 2/16	6.5	7.0	Positive
3/12/17	11.5	12.5	Positive
	11.5	12.5	Positive
3/13/17	11.5	11.0	Positive
	11.0	11.0	Positive
	11.0	11.0	Positive
	11.0	11.0	Positive
	11.0	11.0	Positive. Not marked. Demonstrated
3/15/17	10.0	10.0	Positive
3/16/17	10.0	10.0	Positive
3/20/17	11.0	11.0	Positive
3/21/17	11.0	11.0	Positive
3/22/17	10.0	10.0	Positive

Number tested 23 pairs.   Positive more, 21.   Negative more, 2.

Chart 1   Showing graphically the reaction of six untreated nymphs to light from a 100-watt daylight Mazda (C2) placed 50 cm. from the experimental dishes. The charting was done by an assistant from whose records these graphs were copied.   The scales show time in minutes.   The left-hand side represents the positive end of the dish, i.e., the end toward the light.   Where the line is approximately straight vertically the nymph was resting quietly.   Curves and kinks show movement which did not markedly change the position in the dish in respect to light.   Column 1 gives the reactions of a nymph that was predominantly positive to light, which after twenty-three minutes' exposure gave more carbon dioxide in the biometer than the negative nymph whose reactions are recorded in column 2.   Column 3 shows a nymph made positive by long exposure to light. In this case the reversal came suddenly.   Column 4 gives the same result obtained by a different method.   Perhaps column 3 represents a 'tropic' and column 4 a 'trial' reaction.   Columns 5 and 6 show the reactions of two nymphs that were tested in the biometer before exposure to light.   The animal with the higher rate of carbon dioxide production became positive.

TABLE 6
Showing the effect of long exposure to light upon carbon dioxide production

SIZE		TEMPERATURE		MINUTES EXPOSED	MORE CARBON DIOXIDE
Exposed	Unexposed	Exposed	Unexposed		
mm.	*mm.*	*deg C.*	*deg C.*		
13	13	21	19	120	Exposed
13	14	21	19	150	Exposed
10	10	23	21	204	Exposed
10	10	23	21	234	Exposed
10	10	15	13	52	Exposed

EXPERIMENTS WITH HYDROCHLORIC ACID

1. Effect on negative nymphs

As with the other species studied, hydrochloric acid was one of the most effective reagents in causing reversals. In one set of carefully controlled, fully plotted experiments there were a total of 220 control or preliminary readings lasting from fifteen to ninety-three minutes. Of the nymphs thus tested, thirty-seven were or became positive without other treatment than exposure to light. This is 17 per cent of the number tested.

In this same series of experiments 125 nymphs that had been consistently negative through a preliminary testing period of at least fifteen minutes, were treated with N/25 hydrochloric acid. Under this treatment, seventy-five nymphs, 60 per cent, became positive. Of the fifty nymphs that did not reverse, twenty-five were kept under observation for less than twenty-five minutes and only two were kept until they died. If it had been the purpose of the experiments to ascertain how many nymphs could be reversed by the treatment, doubtless about 90 per cent would have become positive before death resulted.

The typical effect of the acid upon the light reactions of these nymphs is shown in chart 2. The graphs show that the nymphs may reverse soon after being placed in the acid or the reversal may come only after long exposure. Forty-eight per cent of the reversals came within fifteen minutes, but reversals occurred after seventy minutes' treatment. Death frequently followed close upon the later type of reversals.

The effect of this treatment upon the carbon dioxide production is shown in table 7, which lists all the determinations, and in table 8, which partially analyzes the results listed in the preceding table.

The biometer tests show that the nymphs were stimulated when first put into the acid and that this period of stimulation lasted approximately fifteen minutes. The time limits varied with different individuals. After this period of stimulation the nymphs were depressed. This gives two periods in the carbon dioxide production corresponding to the two periods in the reversals by this strength of the acid.

The tables show that all nymphs were not tested immediately after reversal, but of those whose carbon dioxide production was found within two minutes after reversal and which had been stimulated by the acid the average time of treatment was 13.2 minutes. Thirteen of the twenty-five nymphs so tested had reversed within ten minutes after being placed in the hydrochloric acid. On the other hand, the average time of treatment of the animals similarly reversed and measured, but giving more carbon dioxide in the control than in the acid, was twenty-seven minutes, and seven of the seventeen nymphs reversed after twenty-seven to thirty-seven minutes' treatment.

From these experiments it appears that either a marked increase or a decrease may accompany phototactic reversals of these nymphs when treated with hydrochloric acid.

2. Effect on positive nymphs

About 20 per cent of the Heptageninae tested were positive to light when first tested or became positive under the influence of exposure to light for a short time. This positive reaction is much less stable than the usual negative reaction. Of fifteen carefully plotted tests lasting from 15 to 114 minutes, seven, or 47 per cent, showed a change to the usual negative reaction without treatment. In nineteen tests lasting from 1 to 68 minutes seventeen of the nymphs, 89 per cent, were made negative by N/25 hydrochloric acid. Most of these became negative within the first five minutes of treatment and, as was to be expected from the preceding

Chart 2

1 + 6/15/17 −
2 + 6/15/17 −
3 + 5/18/17 −
4 + 5/18/17 −
5 + 5/28/17 −
6 + 5/28/17 −

Water Water Water Water Acid Water

9 1/2 minutes omitted

Changed to N/25 HCl
Changed to water

More CO_2 Less CO_2

N/25 HCl 5/26/17
Water 5/26/17
Changed to water
Changed to N/25 HCl

More CO_2 Less CO_2 Less CO_2 More CO_2 19' omitted Less CO_2 More CO_2
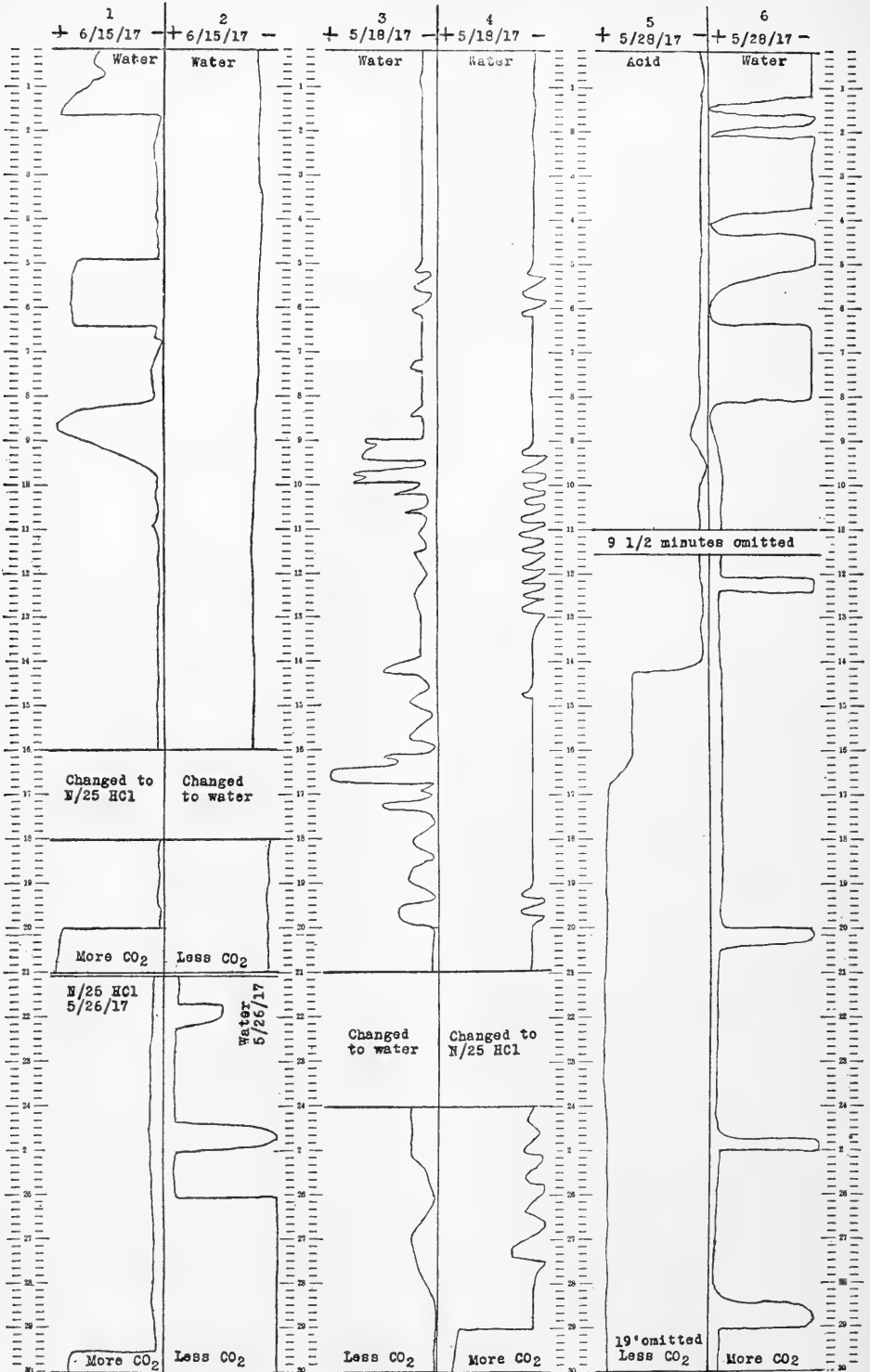
experiments, they were found to be stimulated by the treatment (table 9).

Two instances of the reversal of positive animals by acid treatment are given in the first two columns of chart 4, p. 450.   In both instances shown the reversals are typical in that they are very marked and occur almost directly upon the start of acid treatment.   In the second instance shown the experimental dish was twice turned end for end and each time the nymph moved directly negative.

Here we have the same reagent making negative animals positive by either stimulating or depressing them and making positive animals of the same species negative by stimulating them.

3. Negative vs. positive nymphs; both treated with HCl

The treatment with hydrochloric acid did not cause all the nymphs to reverse their light reactions.   When animals that had been made positive were compared with those still negative, it was found (table 10) that the former had a higher rate of carbon dioxide production when the time of exposure had been approximately the same for both nymphs compared.   Almost all the nymphs tested came from the preliminary period of stimulation. Theoretically, nymphs long exposed to acid and positive would give less carbon dioxide than those more recently placed in the acid and still negative.   This possibility was not tested, since the results can easily be interpolated from the tests given in previous   tables.

Chart 2 Showing graphically the light reactions of four nymphs treated with N/25 HCl and their carbon dioxide production as compared with that of their control nymphs. Column 1 shows two reversals with the acid treatment, the first of which occurred within three minutes.   The reversals shown in column 1 and that in column 4 were accompanied by stimulation. On the other hand, the reversal shown in column 5, which came after twenty-three minutes' exposure to the acid and which was allowed to remain positive for thirty-five minutes before testing, showed depression. The preliminary test in columns 5 and 6 is not charted, but was essentially like the first eight minutes shown.   It will be noted that in this test the control became positive due to exposure to light and was therefore more stimulated than the ordinary negative control. Other tests show depression by treatment with acid for this length of time when compared with negative nymphs.

TABLE 7

Showing the effect of N/25 hydrochloric acid upon carbon dioxide production. All the nymphs here listed were decidedly negative in their light reaction before being placed in the acid

SIZE		SIGN OF LIGHT REACTION		MORE CO₂	MINUTES IN HCl	MINUTES BETWEEN REVERSAL AND TEST
+	−	HCl	Control			
13	13	+	−	Acid	2	1
13	13	+	+	Acid	2	1
11	11	+	−	Acid	2	1
13	13	+	−	Acid	3	1
13	13	+	−	Acid	5	2
13	13	+	−	Acid	5	2
12	12	+	−	Water	5	2
11	11	+	−	Water	6	1
10	10	+	−	Acid	6	1
10	10	+	−	Acid	6	1
11	11	+	−	Acid	6	1
12	12	+	+	Acid	7	1
10	10	+	−	Acid	7	1
11	11	+	−	Acid	8	1
10	10	+	−	Acid	10	1
10	10	+	−	Water	10	7
11	11	+	−	Acid	12	1
11	11	+	−	Acid	12	3
12	12	+	+	Acid	12	1
13	13	+	−	Water	12	1
13	13	+	−	Acid	13	2
13	13	+	+	Acid	14	2
11	11	+	−	Water	14	4
15	15	+	−	Water	15	15
13	13	+	+	Acid	16	1
14	14	+	+	Water	17	1
13	13	+	−	Acid	17	1
13	13	+	−	Water	17	1
13	13	+	−	Water	18	1
13	13	+	−	Acid	18	1
12	12	+	−	Water	19	1
11	11	+	−	Water	20	12
11	11	−	−	Acid	21	0
12	12	+	+	Water	22	1
14	14	+	−	Acid	23	1
11	11	−	−	Acid	23	0
14	14	+	−	Water	28	1
13	13	+	−	Water	28	5
10	10	+	+	Water	28	7

TABLE 7—*Concluded*

SIZE		SIGN OF LIGHT REACTION		MORE CO₂	MINUTES IN HCl	MINUTES BETWEEN REVERSAL AND TEST
+	−	HCl	Control			
10	10	−	−	Water	29	0
11	11	+	−	Acid	30	5
11	11	+	−	Water	31	6
13	13	+	−	Water	33	1
10	10	−	−	Water	33	0
12	12	+	−	Water	33	1
12	12	+	−	Water	35	1
12	12	+	−	Water	37	1
15	13	+	−	Water	37	1
13	13	+	−	Water	38	38
13	13	+	−	Water	41	1
13	13	+	−	Water	41	14
11	11	+	−	Water	42	33
13	13	+	−	Water	44	1
11	11	−	−	Acid	44	0
10	10	−	−	Acid	48	0
13	13	+	−	Water	51	14
13	13	+	−	Water	58	3
13	13	+	−	Water	59	7
11	11	+	−	Water	67	0
13	13	+	+	Water	70	30
11	10	+	−	Water	78	10

TABLE 8

Showing relative carbon dioxide production of experimental and control nymphs analyzed on the basis of length of exposure to hydrochloric acid. This table is based on table 7

MINUTES IN HCl	MORE CARBON DIOXIDE	
	HCl	Water
Part I. Showing all tests listed in table 7		
1– 5	6	1
6–10	8	2
11–15	5	3
16–20	2	5
21–25	3	1
26–30	1	4
31–40	0	7
41–50	2	4
51–60	0	3
61–70	0	2
71–80	0	1

TABLE 8—*Concluded*

MINUTES IN HCl	MORE CARBON DIOXIDE	
	HCl	Water

Part II. Showing results of the biometer tests made within two minutes after reversal

MINUTES IN HCl	HCl	Water
1– 5	6	1
6–10	7	1
11–15	5	1
16–20	2	3
21–25	3	1
26–30	1	2
31–40	0	5
41–50	1	2
67	0	1

TABLE 9

Showing the effect on carbon dioxide production of treating positive nymphs with N/25 hydrochloric acid until they became negative

SIZE		SIGN OF LIGHT REACTION		MORE CO_2	MINUTES IN HCl	MINUTES BETWEEN REVERSAL AND TEST
HCl	Control	HCl	Control			
13	13	—	—	Water	2	2
12	12	—	—	Acid	3	3
13	13	—	—	Acid	3	2
13	13	—	—	Acid	3	3
10	10	—	+	Acid	3	2
12	12	—	—	Acid	4	1
13	13	—	—	Acid	4	4
14	14	—	+	Acid	9	1

TABLE 10

Showing the relative carbon dioxide production of negative nymphs made positive by treatment with N/25 hydrochloric acid, compared with those similarly treated but not reversed

SIZE		MORE CO_2	MINUTES IN HCl		MINUTES BETWEEN RE-VERSAL AND TEST
+	—		+	—	
13	13	+	2	2	1
14	14	+	4	4	2
12	12	+	3	3	1
14	14	+	10	10	1
12	12	+	13	13	13
14	13	+	14	14	3
12	12	+	15	15	3
12	12	+	15	15	6
11	11	+	25	25	2
11	11	Both same	32	29	3
11	11	+	9	21	2
13	13	+	15	15	2

EXPERIMENTS WITH POTASSIUM CYANIDE

In the experiments with the negative Leptophlebia potassium cyanide had proved an effective reagent in causing reversals. With these Heptageninae at a concentration of $N/500$ it was almost as effective in causing reversals as hydrochloric acid. Of thirty tests run, 57 per cent were reversed by the cyanide while the controls showed no reversals at all. Biometer tests proved that the cyanide clearly depressed the nymphs. The details are listed in table 11 and specimen results are shown in chart 3. In all cases the nymphs were washed in water after treating with KCN in order to keep the potassium from interfering with the CO_2 determination.

In addition to the carbon dioxide tests, one nymph died while moving positive, another just after reaching the positive end. In all ten nymphs died in the experimental dishes as a result of the cyanide treatment, of these six had reversed their reaction to light shortly before dying.

TABLE 11

Showing the effect of $N/500$ potassium cyanide upon nymphs negative to light and upon their rate of carbon dioxide production

SIZE		SIGN OF LIGHT REACTION		MORE CO_2	MINUTES IN KCN	MINUTES BETWEEN REVERSAL AND TEST
KCN	Water	KCN	Water			
13	13	+	—	Water	3	2
12	12	+	—	Water	4.5	1
14	14	+	—	Water	6	3
13	13	+	—	Water	7	2
12	12	+	—	Water	8	2
13	13	+	—	Water	6	1
13	13	+	—	Water	9	3
13	13	+	—	Water	14	4
13	13	+	—	Water	16	5
12	12	+	—	Water	19	2
12	12	+	—	Water	41	1

Chart 3

448
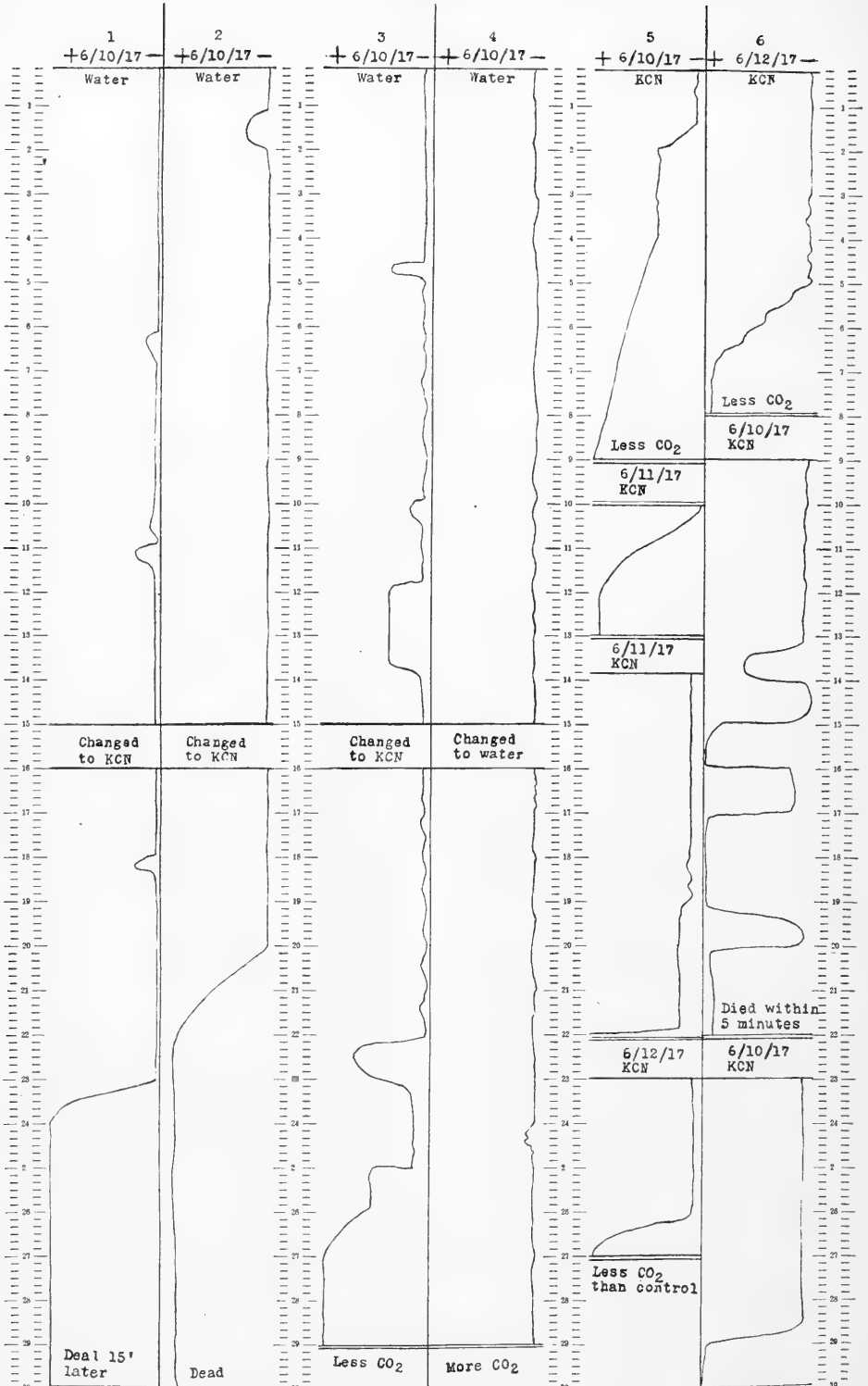
EXPERIMENTS WITH ALCOHOL AND STRYCHNINE

Ethyl alcohol had less marked effect in causing reversals among these Heptageninae than with the species used in the first part of the work.   In twenty-five well-studied cases, only five nymphs were by treatment with 2 per cent solution reversed.   Of the fifty-two accompanying preliminary or control readings, one nymph became positive.   When alcoholic nymphs were tested in the biometer (table 14), it was found that alcohol acts here according to its usual effect as a narcotic, first stimulating and later depressing.

Some typical results obtained with the alcohol treatment are given in chart 4.   This shows two tracings of the reactions of nymphs that were not reversed by the treatment and of one that was, together with a type of control reaction which occurred frequently.

Some preliminary experiments with strychnine showed a marked increase in the activity of the nymphs without a reversal of their light response.   The animals usually remained at the negative end of the dish in almost continual motion.   In the biometer, they usually showed a markedly increased carbon dioxide production.

I am not able to state why these nymphs did not reverse their light reactions, but offer these results with alcohol and strychnine as evidence that all stimulation and depression do not cause reversals in phototaxis.

Chart 3   Showing the light reactions of ten nymphs treated with potassium cyanide together with three preliminary test periods and one complete control. In the cases where the preliminary and control graphs are not given it is understood that they are in no essential respect different from those shown.   In order to economize space the time spent in changing from water to cyanide is not fully shown as it was in chart 2.   Where such a change is indicated it must be understood that two or three minutes elapsed.   In all cases given in columns 5 and 6 the control, with which the carbon dioxide production of the treated nymphs was compared, is not given in the chart.   For text reference see p. 535.

Chart 4

1	2	3	4	5	6
6/11/17	6/11/17	4/28/17	4/27/17	4/20/17	4/20/17
Water	Water	Water	Water	Alcohol 15' omitted	Water 15' omitted

Changed to N/25 HCl — Changed to water — Changed to Alc.2%

More CO_2 — Less CO_2 — Changed to Alc.2%

Water 8' omitted — Water 18' omitted

6/12/17 — 6/12/17

HCl N/25

Reversed

Reversed

Less — More CO_2 — More CO_2 than control — More CO_2 than control — Less CO_2 — More CO_2
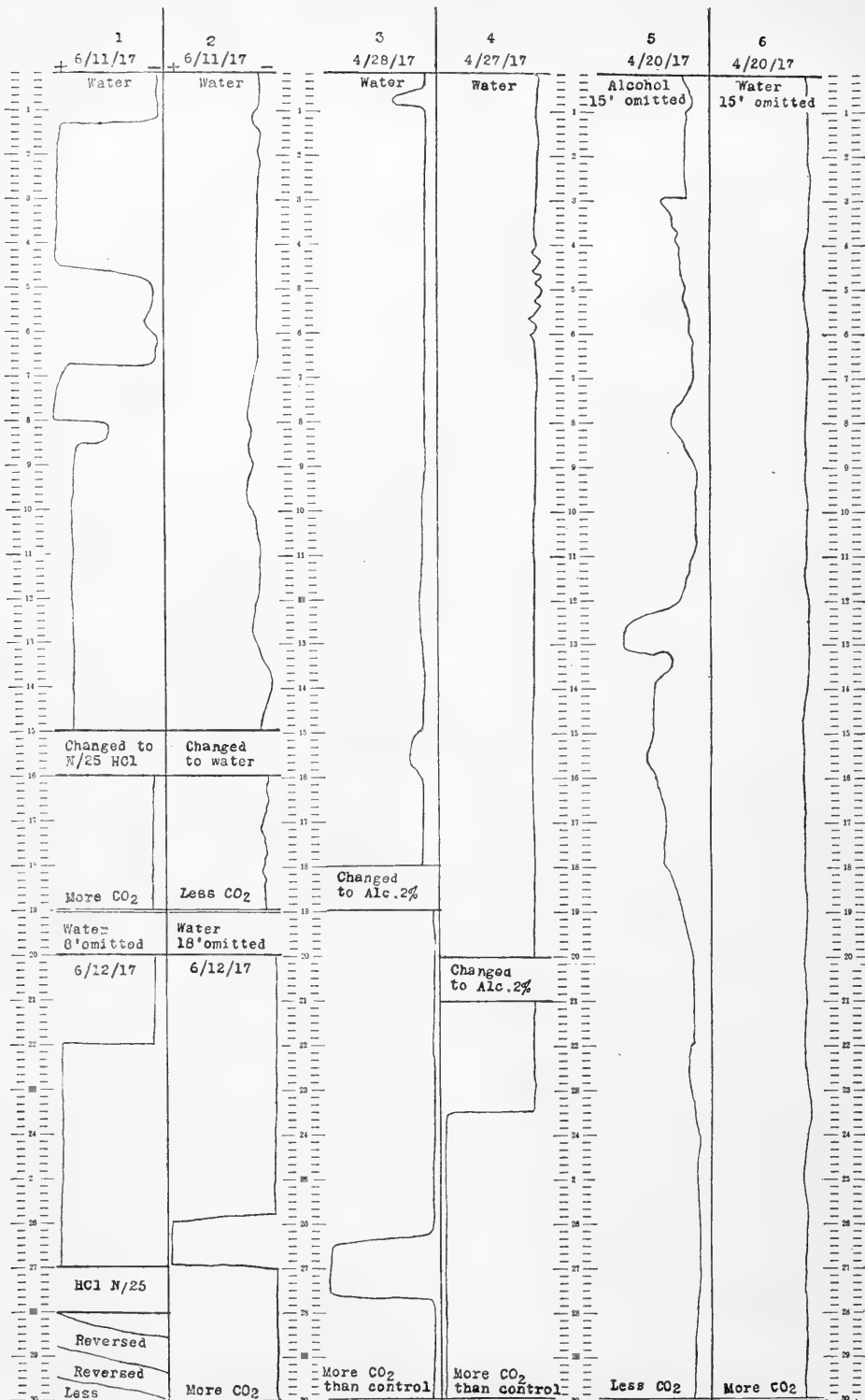
TABLE 12

Showing the effect of 2 per cent ethyl alcohol upon the phototactic reaction of nymphs negative to light and upon their carbon dioxide production

SIZE		SIGN OF LIGHT REACTION		MORE CO_2	MINUTES IN ALCOHOL
Alcohol	Water	Alcohol	Water		
10.5	10.5	+	+	Alcohol	7
8.5	8.5	+	+	Alcohol	8
12.0	12.0	−	−	Alcohol	9
9.0	9.0	−	−	Alcohol	9
11.0	11.0	−	−	Water	9[1]
9.0	9.0	−	−	Water	9[1]
8.0	8.0	−	−	Alcohol	10
11.5	11.5	+	+	Alcohol	10
10.0	10.0	−	+	Water	10[1]
10.5	10.5	−	−	Water	10[1]
11.0	11.0	+	+	Alcohol	11
9.0	9.0	−	−	Alcohol	12
11.0	11.0	−	−	Alcohol	12
11.0	11.0	−	−	Alcohol	13
9.0	9.0	−	−	Alcohol	16
10.5	10.5	+	−	Alcohol	28
10.0	10.0	−	−	Water	44
11.5	11.5	−	−	Water	50
8.5	8.5	−	+	Water	50
10.5	10.5	−	−	Alcohol	52
11.0	11.0	−	−	Water	55
10.0	10.0	−	−	Water	83
9.0	9.0	−	−	Water	180

[1] These control nymphs were more active than the experimental ones before the biometer test was made.

Chart 4   Showing the reactions of two positive nymphs to light after treatment with N/25 hydrochloric acid together with their controls and the result of treating three nymphs with 2 per cent ethyl alcohol with one control tracing. The control shown in column 6 remained quietly at the negative end during almost all of its exposure and yet gave more carbon dioxide than the more active alcohol treated nymph whose response is shown in column 5.   The nymph shown at the bottom of column 1 became negative immediately upon treatment with the acid. The experimental dish was then twice turned end for end each time the nymph moved directly away from the light.

DISCUSSION

Since this is an inquiry into the problem of a possible relationship between the sign of the phototactic reaction and the general rate of metabolism of May-fly nymphs, it is pertinent to question the effectiveness of our means of measuring the rate of the metabolic processes. The cyanide-resistance method is best checked by comparing results obtained with it with those given by Tashiro's method of determining carbon dioxide production. This has been done for the isopod Asellus communis (Allee and Tashiro, '14). There it was found that the two methods gave the same results in direct tests and that two isopods subjected to daily variations of oxygen tension for ten successive days with daily quantitative estimations of carbon dioxide production by Dr. Tashiro behaved according to expectation based upon previous experience with the cyanide method.

Regarding the work with Tashiro's biometer in measuring the carbon dioxide production, I consider this the best, though by no means the fastest, method of making such comparative carbon dioxide tests as those recorded in this paper. It is less complicated than the electrolytic determination of the hydrogen ion concentration and more accurate than the colorimetric methods. The instrument is somewhat complicated in appearance, but it is in reality as simple in manipulation as a modern microscope equipped with oil-immersion lens, mechanical stage, and camera lucida. I have repeatedly demonstrated end points to coworkers at Woods Hole and even to college freshmen. My only change from Tashiro's technique ('17, p. 109) consists in the use of a low-power binocular in reading end points.

The sources of error in the method as applied to May-fly nymphs are:

1. May-fly nymphs are water-dwelling animals and were tested in as nearly a dry atmosphere as possible.

2. Five minutes or more intervened between the time the nymphs were taken from the water and the first indication of the relative rate of carbon dioxide production. During this interval the nymphs must be picked up, partially dried, and placed in glass containers.

Regarding these points it must be borne in mind that the readings here given are all comparative ones in which the control and experimental nymphs were treated exactly alike.   Since one must needs be taken from the water before the other, this was varied in the different experiments.   The nymphs often lived twenty-four hours in the apparatus and at times they lived as long as forty-eight hours in the slight amount of moisture present.

3. Difference in carbon dioxide production may be due to difference in movement.   This source of error was eliminated by the simple method of throwing out all tests where movement occurred.

4. Unconscious personal preference for one of the nymphs producing more carbon dioxide.   This was eliminated by the experimenter's ignorance of which was experiment and which was control.

The relationship between the general metabolic processes of animals and their reaction to light may conceivably be one of the following:

1. Conditions that depress positive animals may make them negative (Mast, Bohn, Drzewina) and conditions that stimulate negative animals may make them positive (Holmes, Carpenter, Bohn, Jackson).

2. The above relationship may be reversed (Phipps in part).

3. Conditions that stimulate animals may cause reversals of their normal reaction or vice versa.

4. Conditions that markedly change the metabolic rate may cause reversal of either positive or negative animals.

5. Changes of metabolism accompanying changes in light reactions may be incidental or resultant rather than causal.

6. The relation between metabolic processes and the reaction to light may vary in different species so that no general law can be worked out.

7. There may be no relationship between the rate of metabolism and the phototactic reaction.

With the positive Epeorus nymphs only depressing agents were used and these caused reversals.   With both the negative

species both stimulation and depression resulted in reversal
and in positive members of the Lake Forest Heptageninae stim-
ulation of positive nymphs caused reversal. In Leptophlebia
thirty-six tests with hydrochloric acid showed no effect on the
average resistance to potassium cyanide. The biometer tests
with the Heptageninae give the explanation. When first sub-
jected to the acid the nymphs are stimulated; later they are de-
pressed. If taken during the first period the resistance to cya-
nide would offset that of the later period and so yield an average
about the same as the control. An examination of the carbon
dioxide production records shows that about as many nymphs
were stimulated as were depressed by the treatment, so that a
general average not considering the time factor would show re-
versals with no relation to the rate of carbon dioxide production.

In the May-fly nymphs studied the results obtained demon-
strate that there is a relationship between the rate of metabolic
processes and the sign of the phototactic reaction. It is also
clear that reversals are accompanied by either a marked stimula-
tion or marked depression. The experiments indicated but do
not prove that the stimulation or depression is causal. From
one point of view it makes little difference whether the metabolic
changes are causal or only symptomatic; the fact that they are
correlated at all is important.

If the change in metabolic conditions is causal, the fact is evi-
dent that all changes do not cause reversals. This was shown
particularly by the action of the ethyl alcohol upon the Lake
Forest nymphs. This stimulated and later epressed the
nymphs with or without an accompanying reversal. On the
assumption that metabolic change is causal the non-action
of alcohol in 80 per cent of the cases might be explained by
supposing that it did not cause a change quantitatively large
enough. This suggestion is supported by the observation with
the other species that alcohol accompanied by decrease in tem-
perature ws more effective in producing reversals than alcohol
alone and by the fact that in general these species were more
susceptible.

The idea that a certain quantitative change must occur before reversal in light reaction takes place is also supported by carbon dioxide determinations made before the nymphs were exposed to light. In some of these, as, for example, the results shown in columns 5 and 6 of chart 1, the nymph with the higher speed of carbon dioxide production became positive upon exposure to light while the other was decidedly negative. It more frequently happened that both nymphs so treated were negative in spite of the fact that one had a higher rate of metabolism than the other. Evidently the biometer is more sensitive to changes in carbon dioxide production than is the light reaction.

Strychnine, again, did not cause a high degree of reversals, but it did strongly stimulate the nymphs. There is no question but that this stimulation is as strong as that caused by hydrochloric acid which caused a high percentage of reversals. This difference in result can only be explained by the assumption that while light reversals are accompanied by changes in metabolism that are probably causal, all such changes do not cause reversals in reaction to light.

The untreated positive nymphs were found to give off more carbon dioxde than untreated negative ones. These negative animals often moved back and forth in the dishes, spending the major part of the time at the negative end. Some nymphs do this more than others. This brings them to the positive end more frequently and gives them more opportunities to come to rest there. A reversal apparently on this plan is shown in column 4, chart 1. It appears that this is one mechanism for reversal to light and here the relation between metabolic rate and the reversal is obvious. The higher the metabolic rate, the greater the tendency to move back and forth; the more random movements, the greater the chance of becoming acclimated to the positive end and of reversing the normal reaction.

All eversals even under the influence of light alone were not of this type, witness column 3, chart 1. This reversal, like most of those experimentally obtained by the use of chemicals, was not preceded by random movements, but took place as though the animal had suddenly discovered the attractiveness of the positive end and must needs go there even though it died in the attempt

(as in cyanide reversals). This excursion was often the first one the nymph had made to the positive end and frequently the nymphs remained there until they died. The reason for the relation of metabolic rate with this type of reversal is not evident, especially when the reversal followed a very strong depression.

An explanation of reversals sometimes advanced (Ewald, '13) is that animals change in their sensitivity to light and hence reverse their reactions. On this basis the change in sensitivity is causal; the light reversal and change in carbon dioxide production, resultants. In May fly nymphs these supposed resultants are correlated in such a way that either stimulation or depression may accompany reversals from negative to positive light reactions. This means that either increasing or decreasing sensitivity to light will make negative May-fly nymphs positive, or that an increase (or decrease) in sensitivity wil. cause now depression and now stimulation. Either of these necessary assumptions expand the sensitivity hypothesis beyond the limits justified by known facts.

CONCLUSIONS

The light reactions of the positive May-fly nymph, Epeorus, was reversed by treatment with alcohol, lowered temperature, calcium chloride, and other reagents. Nymphs so reversed had a lower rate of metabolism, as measured by resistance to potassium cyanide, than normal untreated nymphs.

The negative nymph, Leptophlebia, was similarly reversed with accompanying stimulation or depression as measured by resistance to the cyanide.

A negative nymph belonging to the Heptageninae was reversed in its light reactions with accompanying increase or decrease in carbon dioxide production as measured by Tashiro's biometer.

The experiments conclusively demonstrate that the phototactic reaction of these nymphs is correlated with the metabolic condition and indicate, but do not prove, that certain changes in metabolism cause the reversals in reaction to light.

All nymphs that reversed their light reactions we e e ther stimulated or depressed, but stimulation or depression did not necessarily involve phototactic reversal.

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STUDIES ON THE PHYSIOLOGICAL SIGNIFICANCE OF CERTAIN PRECIPITATES FROM THE EGG SECRETIONS OF ARBACIA AND ASTERIAS

ALVALYN E. WOODWARD

Simmons College, Boston, Massachusetts

TWO CHARTS AND THREE FIGURES

CONTENTS

I. INTRODUCTION

A new phase in the physiology of fertilization practically began in 1912 when F. R. Lillie discovered the effect of egg extracts and secretions on sperm. He found that sea-water in which Arbacia eggs had been standing caused sperm of the same species to be activated, directed, reversibly agglutinated, and finally paralyzed. If a drop of this egg-sea-water is introduced, by means of a

capillary pipette, into a suspension of sperm, the latter become more active and gather around the drop as if chemically attracted. In addition, they form small clusters, 'agglutinations,' which last for several seconds. After this the sperm separate. The period of agglutination depends upon the activity and density of the sperm suspension and the strength of the egg-water. The effective material in the water Lillie ('12) called an iso-agglutinin.

The eggs of Nereis were found to produce a similar substance, which seemed to appear at the moment of fertilization. In this case, however, the active substance is destroyed by boiling, while Arbacia agglutinin is not entirely destroyed when boiled for seventy minutes. Lillie also found that an extract of Arbacia eggs agglutinated Nereis sperm. Because of differences in lability, he concluded that the Arbacia egg produced both iso- and hetero-agglutinins. He also discovered that if sperm be added in quantity to egg-water, the agglutinin is 'fixed,' and no longer affects sperm subsequently added.

For purposes of quantitative comparison, Lillie prepared a 'standard solution' of egg secretion by allowing one volume of 'dry' ripe eggs to stand in two volumes of sea-water for ten minutes. During this time the eggs were occasionally agitated. They were then precipitated with the centrifuge, and the supernatant fluid, carefully decanted, was subsequently used (Glaser, '14 c, p. 388). Since this secretion had such peculiar effects on spermatozoa, and since eggs from which it had been removed by repeated washings could not be fertilized Lillie has since called it 'fertilizin' ('13 b).

A substance to which the cell membrane is not permeable was also found in the egg. This material could be obtained only by laking with distilled water or crushing. Since this material was able to neutralize the agglutinative power of fertilizin, Lillie called it 'anti-fertilizin.' Antifertilizin does not prevent the chemotactic effect of egg secretion.

In addition, Lillie likewise discovered that Arbacia blood inhibits fertilization in the same species, but does not affect the agglutinative power of fertilizin. Moreover, the inhibitory effect of blood can be counteracted by an excess of fertilizin.

From these facts, Lillie deduced his 'side chain' theory of fertilization, in which he applied Ehrlich's conceptions to the subject at hand. He postulates that spermatozoa are chemically unable to unite directly with the egg, but that a third body, or amboceptor—in this case, fertilizin—is necessary to join them. Fertilizin, he believes, has two side chains, an ovophile group which can unite with a side chain of the egg, the egg receptor, and a spermophile group, which can unite with a sperm receptor. Fertilization may be blocked by 1) absence of the amboceptor; 2) occupation of either side chain by a foreign body; 3) occupation by similar means of either sperm or egg receptor. Further, "The sperm activates the fertilizing substance (fertilizin) already present in the egg. The egg is self-fertilizing (Lillie, 14, p. 587)."

II. CONCERNING THE FACTUAL BASIS OF THE FERTILIZIN THEORY

The fertilizin theory is founded upon three essential elements: an amboceptor, an egg receptor, and a sperm receptor. The presence of the amboceptor, Lillie believes, is indicated by a sharply defined reaction-agglutination of the sperm. This reaction is considered ('15) only as a visible, but not essential, symptom of a fundamental physicochemical change in the spermatozoa themselves. It is possible that in many species the egg secretion produces a significant change in the sperm without agglutinating them. The presence of sperm receptors, according to the same author, is indicated, among other things, by the Godlewski phenomenon, in which the inhibitory effect of foreign sperm is explained as due to the occupancy of the normal sperm receptors. The presence of an egg receptor he has been unable to demonstrate directly. It is, however, indicated by other inhibitors which effectively block the fertilization reaction.

A. Concerning egg secretions in general

The observations of Lillie with regard to the egg secretions of Nereis and Arbacia have been extended by several workers upon the same and other forms.

Just ('15 a) found that it was difficult or impossible to initiate development in Nereis eggs which had been washed. The eggs of Platynereis, also, are very sensitive to an excess of sea-water ('15 b). From these facts he concluded that a substance formed by the egg and necessary for development had been removed.

Fuchs found that the fertilizing power of the sperm of Ciona, Arbacia pustulosa, Ascidia mentaul, and Strongylocentrotus lividus could be increased by treatment with egg secretions of the same species. Moreover, the fertility of Ciona sperm could be enhanced by treatment with the egg secretion of Phallusia, Arbacia, or Stronglyocentrotus. Likewise, Strongylocentrotus sperm are made more effective by the secretions of Ciona, Echinus, Sphaerechinus, and Asterias eggs. The egg extract, obtained by crushing the eggs, had the same effect as the secretion, except in the case of Asterias. In this instance, the secretion stimulates, while the extract poisons, Strongylocentrotus sperm. The method followed by Fuchs prevented the observation of sperm agglutination. He concluded that the secretions affected sperm only.

Asterias eggs, Glaser found ('14 b), form a secretion similar to that of Arbacia, except in color and a few minor points. Moreover, Asterias sperm are directed, activated, agglutinated, and paralyzed as well by Arbacia secretion as by that from their own species. Arbacia sperm react similarly to Asterias secretion. The writer has been able to confirm these observations and to demonstrate the reactions to colleagues.

While mature Arbacia eggs in a healthy condition are easy to obtain and always secrete a sperm agglutinin, the conditions differ in the case of Asterias. It was difficult, during the summers of 1915 and 1916, to obtain Asterias which contained many fertilizable eggs. In a good batch 10 per cent would produce larvae. Therefore, it was difficult to obtain the agglutinin in any usable amount. In June, 1914, however, Asterias at Woods Hole were in the best condition I have ever known. It was almost impossible to keep them in the laboratory three or four hours without their shedding eggs or sperm. Mr. Gray reported that the livecars in which they were kept, though very open, were always

slimy with eggs.   At that time, when 98 per cent to 100 per cent of the eggs would develop normally, most of the observations on the sperm agglutinin were made.   All of them, however, were repeated and confirmed in 1915, 1916, or 1917.

As observed by Glaser, if a drop of sea-water, which had been standing over mature Asterias eggs, was injected under a cover-glass into a drop of sperm suspension, the sperm would gather into small, irregularly angular clusters of six to eighteen and remain agglutinated for a number of seconds which varied with the strength of the solution.   When they separated, they were much more active than before.   The general process of agglutination and activation is the same in Asterias as in Arbacia, where it has been observed by many.   The chief difference is in the size of the clusters, which are smaller in Asterias.

As is known, Asterias eggs are obtained with the germinal vesicle still intact.   Maturation changes begin almost immediately and are complete in forty-five to sixty minutes.   Directly after obtaining them, some of the eggs of a large female were put into a centrifuge tube with two volumes of water and allowed to stand ten minutes to obtain fertilizin in the standard way. After centrifuging, the supernatant fluid was tested for its agglutinative power.   When diluted 1 : 200, Asterias sperm remained agglutinated six seconds, a unit reaction.   An hour after shedding, the rest of the eggs from the same female, which had been kept in a large amount of water, were washed twice with fresh sea-water, and then the secretion obtained as before.   This, when diluted 1: 200, caused a fresh sperm suspension to remain agglutinated over four minutes.   In other words, it was over sixty times as strong as the other.   Experiments of Lillie and Just indicate that in Arbacia and Nereis, as well as in Asterias, fertilizin is formed by the maturing or mature, but not by immature eggs.   I believe that Lillie's failure (quoted by Loeb, '16, p. 83) to verify the observations with Asterias secretion was due to abnormal material.

Loeb ('15) has observed activation of sperm of the echinoderms at Pacific Grove by eggs of the same and related species.   He also noted that the sperm of Strongylocentrotus purpuratus are

agglutinated by the egg secretion of the same species, while sperm of S. franciscanus are agglutinated not only by secretion from eggs of the same species, but also by that of S. purpuratus.

Miss Margaret V. Cobb (unpublished) discovered that the eggs and egg-water of Cumingia produce positive chemotactic response on the part of the sperm.

These results are summarized in table 1 and warrant the assertion that the eggs of at least four phyla of marine animals secrete into the sea-water substances significant either in their effects on sperm or in fertilization.

B. *Concerning the secretions of Asterias and Arbacia in particular*

While these facts indicate that the egg secretion may play an important rôle in fertilization, they do not make clear either the nature of the secretion itself or the manner in which it operates. It may be one homogeneous substance or a mixture of two or more specific substances.

Conceivably, there are three ways in which the problem can be attacked: 1) by physiological analysis of the secretion; 2) by general chemical analysis; 3) by the removal of specific elements from the secretion and the further analysis of their individual properties.

1. *Physiological analysis.* a. The secretion is necessary for fertilization in some species. As stated above, Lillie gave the name 'fertilizin' to the egg secretion because he considered its presence absolutely necessary for the fertilization of Arbacia eggs. Just found it equally indispensable for the fertilization of Nereis and Platynereis.

Loeb ('15) criticised their conclusion on the ground that the washed eggs had stood so long after shedding that they were dead, but the following experiment disposes of this objection. The eggs from ripe starfish were divided into two lots. One lot, A, was put into a finger-bowl of sea-water as control. The rest were placed into a large glass tube, closed at the bottom with chamois skin. The upper end was similarly closed except for an opening into which fitted closely a smaller tube through which

TABLE 1

SPERM OF THE FOLLOWING SPECIES

WHEN TREATED WITH THE EGG EXTRACT OR SECRETION OF	Arbacia punctulata	Arbacia pustulosa	Ascidia mentula	Asterias forbesii	Asterias ochracea	Asterina	Ciona intestinalis	Cuningia	Echinus microtuberculatus	Nereis limbata	Platynereis megalops	Strongylocentrotus franciscanus	Strongylocentrotus lividus	Strongylocentrotus purpuratus
Arbacia punctulata	Activated, aggregated, agglutinated, paralyzed (Lillie, Glaser, Woodward)			Activated aggregated, agglutinated paralyzed (Glaser, Woodward)						Agglutinated (Lillie)				
Arbacia pustulosa		Become more fertile (Fuchs)					Become more fertile (Fuchs)							
Ascidia mentula			Become more fertile (Fuchs)											
Asterias forbesii	Activated, agglutinated (Glaser, Woodward)													

TABLE 1—*Concluded*

SPERM OF THE FOLLOWING SPECIES

WHEN TREATED WITH THE EGG EXTRACT OR SECRETION OF	Arbacia punctulata	Arbacia pustulosa	Ascidia mentula	Asterias forbesii	Asterias ochracea	Asterina	Ciona intestinalis	Cumingia	Echinus microtuberculatus	Nereis limbata	Platynereis megalops	Strongylocentrotus franciscanus	Strongylocentrotus lividus	Strongylocentrotus purpuratus
Asterias glacialis													Paralyzed by egg extract stimulated by egg secretion (Fuchs)	
Asterias ochracea					Activated (Loeb)	No effect (Loeb)						Activated (Loeb)		Slightly activated (Loeb)
Asterina					No effect (Loeb)	Activated (Loeb)						Activated (Loeb)		Slightly Activated (Loeb)
Ciona intestinalis							Become more fertile (Fuchs)						Become more fertile (Fuchs)	
Cumingia								Chemotaxis (Cobb)						
Echinus microtuberculatus									More fertile (Fuchs)				More cleavage; stimulates hybridization (Fuchs)	

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Species						
Nereis limbata	No effect (Lillie)			Agglutinated (Lillie) Secretion necessary for fertilization (Just)		
Phallusia mamillata						
Platynereis megalops		Become more fertile (Fuchs)		Egg-secretion necessary for fertilization (Just)		
Sphaerechinus granularis		Become more fertile (Fuchs)			Become more fertile (Fuchs)	
Strongylocentrotus franciscanus	Slightly activated (Loeb)	No effect (Loeb)			Activated, agglutinated (Loeb)	Activated, not agglutinated (Loeb)
Strongylocentrotus lividus		Become more fertile (Fuchs)	More cleavage, stimulates hybridization (Fuchs)		Activated, attracted. More fertile (Fuchs)	
Strongylocentrotus purpuratus	Slightly activated (Loeb)	No effect? (Loeb)			Activated, agglutinated (Loeb)	Activated, agglutinated (Loeb)

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sea-water trickled slowly. The eggs were washed in running water in this manner for seventeen hours. These washed eggs were then divided into lots B and C. To B fresh egg secretion was added. All three were subsequently inseminated. In spite of the time which had elapsed since the eggs had been shed, a large number of the control, A, formed membranes. About the same number formed membranes in B, the washed eggs to which the secretion had been added, but in C, which was practically free from fertilizin, none was formed. None of the eggs developed beyond the eight-cell stage, doubtless due to the long time that had elapsed between maturation and insemination. The addition of egg secretion had restored the washed eggs to a fertilizable condition.

If, at the height of the breeding season, the eggs of Asterias or Arbacia are inseminated after the addition of fertilizin, the proportion developing is not materially affected. However, at the beginning or end of the season, a large part of the eggs are resistant to sperm fertilization. If fertilizin be added at that time, it will help to overcome this resistance as shown below:

TABLE 2[1]

The effect of adding fertilizin to normal and to resistant eggs

DATE	ARBACIA EGGS + SPERM	EGGS + FERTILIZIN + SPERM
	per cent cleavage	per cent cleavage
July 2	72	68
	73	73
	84	75
August 9	24	86
	·47	88

The result might be due to an increased permeability, since Glaser found that fertilizin has such an effect. It is very likely, however, that the resistance is due to lack of fertilizin, since egg

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[1] Throughout the experiments, percentages have been reckoned from counts of at least two hundred normal-appearing eggs.

secretion obtained August 11, 1914, tested to only 50-units strength while that obtained by exactly the same method early in the season averaged 1600 to 3200 units and frequently ran higher.

b. The secretion, as stated on page 45, activates the sperm, attracts, reversibly agglutinates, and may finally paralyze them.

c. The secretion has equally striking effects on the egg.   Glaser ('14 c) observed that Arenicola larvae treated with egg secretion lose pigment, indicating an increase in permeability.   Taking his cue from this, and from the fact that parthenogenetic agents as a class increase permeability, he found that when the secretion derived from either Asterias or Arbacia was added to ripe eggs of the same species, and allowed to act on them for two hours, the eggs began to segment.   To this process he gave the name 'auto-parthenogenesis.'   Following the method of Loeb ('13, p. 70), Glaser found that after-treatment with hypertonic sea-water (50 cc. sea-water and 8 cc. 2.5 m. NaCl) increased the percentage of developing eggs.

Glaser's work on autoparthenogenesis was easily confirmed. The 'dry' ripe eggs of Asterias were placed in watch-glasses with at least two volumes of standard fertilizin for one and one-half to three hours.   They were then rinsed two or three times with sea-water, and treated with hypertonic sea-water (50 cc. sea-water and 8 cc. 2.5 m. NaCl) for a suitable length of time.   After rinsing with sea-water, they were left to develop.   It is better to transfer them to finger-bowls with a quantity of sea-water (table 3). The optimum time for the exposure of the eggs to fertilizin and for the hypertonic after-treatment seemed to vary with the temperature and with the batch of eggs.   Occasionally the entire lot of eggs would give normal cleavage and normal gastrulae.   More often, however, cleavage of both nucleus and cytoplasm was irregular, as described by Wilson ('01) for parthenogenetic eggs. If, when the cytoplasm first divided, the nucleus went entirely into one half, the other half would disintegrate and only the nucleated part would develop into a ciliated blastula.   Consequently, it was a frequent occurrence to find a small Asterias blastula swimming around among cytoplasmic fragments within

the fertilization membrane. Practically the same thing occurred in the case of Arbacia, with the exception that no fertilization membrane was formed, and only the jelly surrounding the egg held the fragments together for a time. This soon broke up, however, and allowed the larvae to escape. The irregularity of cleavage was less marked in the eggs which had not been subjected to hypertonic after-treatment, but, on the other hand, the eggs with the hypertonic after-treatment gave a larger per-

TABLE 3

Autoparthenogenesis in Asterias eggs

TREATED WITH FERTILIZIN	HYPERTONIC SEA-WATER	PER CENT CLEAVAGES	REMARKS
hours	*minutes*		
$1\frac{1}{2}$	0	50	1 blastula, cleavage normal
$1\frac{1}{2}$	40	42	Cleavage normal
2	0	50	
2	20	57	Few ciliated
2	30	46	Many ciliated
2	45	41	Few ciliated
$2\frac{1}{2}$	0	52	None ciliated
$2\frac{1}{2}$	20	66	Nearly all ciliated
$2\frac{1}{2}$	30	63	Few ciliated
$2\frac{1}{2}$	45	69	None ciliated
3	0	66	None ciliated
Sperm control..............		74	
Untreated control..........		0	

centage of swimming larvae. That is, these eggs developed farther than those treated with fertilizin alone.

While autoparthenogenesis must be considered in formulating any general theory of fertilization, it is also of immediate practical value in our analysis of the egg secretion, which is thus shown to affect both egg and sperm. This indication that the secretion may have at least a dual nature is strengthened by other observations: 1) Boiling does not destroy its power to agglutinate sperm (Lillie), but does destroy its value as a parthenogenetic agent (Glaser). 2) Arbacia blood inhibits sperm fertilization, but does not affect the agglutinating power of the secretion (Lillie);

in other words, Arbacia blood has no effect on the 'spermophile group' (Lillie). 3) Loeb ('14) dissolved the jelly from the eggs of S. purpuratus by means of $\frac{N}{10}$ HCl. These eggs were no longer able to agglutinate sperm, but could be fertilized. From this experiment and from the fact that some species can be fertilized by sperm of other species, even though those sperm are not agglutinated by secretions from the eggs, Loeb concluded that "The substance which is responsible for cluster-formation is not necessary for the process of fertilization." Lillie ('15) repeated this experiment, using Arbacia instead of Strongylocentrotus, and found, as soon as the acid was entirely washed away, that the eggs continued to secrete an agglutinin. It is possible that in Loeb's experiment the agglutinin was so dilute as to give only a momentary reaction which was overlooked or that the presence of acid interfered with the reaction.

2. *General chemical properties of the egg secretion.* *a.* Properties shown by qualitative chemical tests. Glaser ('14 b) undertook a qualitative analysis of both Arbacia and Asterias secretions. He found them neutral to litmus and unable to reduce Fehling's solution. Various tests for protein failed to give a distinct reaction, although there were indications that protein might be present in very small amounts. For instance, while the "xanthoproteic test gave no precipitate, the solution turned distinctly yellow." He was also unable to obtain a precipitate either by boiling or by adding alcohol.

The writer confirmed all of these points. It was also found that the secretion does not dialyze through a collodion sac. Benedict's test for sugar was applied, as being more sensitive than Fehling's, but gave negative results. Since the xanthoproteic test produces a yellow color, we may infer the presence of tyrosine, phenylalanine, or tryptophane.

It was not deemed advisable to carry out a complete chemical analysis of the secretion. Qualitative tests, however, indicated the presence of both carbon and nitrogen.

b. Properties shown by x-radiation. In 1914 the writer was able, coöperating with A. Richards, to study the effect of x-radiation on Arbacia egg secretion. An account of these experiments,

though already published (Richards and Woodward), may be summarized here. Equal amounts of the secretion were placed in four open dishes. One was kept shielded from x-rays for a control, one was radiated two minutes, another five minutes, and the third, fifteen minutes. The agglutinating power of each was then tested with fresh sperm suspension, by finding the dilution necessary to bring about a unit reaction. It was found that the secretion which had been radiated two minutes had the greatest agglutinating power. That which had been radiated five minutes was about the same as the control, while radiation for fifteen minutes decreased the efficiency of the agglutinin. These

TABLE 4

The effect of x-radiation on the parthenogenetic power of fertilizin

	PER CENT CLEAVAGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAVAGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAVAGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAVAGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAVAGES	PER CENT NORMAL BLASTULAE
Sperm control...................			23.8		46.6					
Fertilizin control (unradiated)....	30.2	0	9.5	0	5.2	1	20.2	.5	15.5	0
Fertilizin 2 minutes radiation....	24.3	Few	14.1	1	10.8	3	13.5	0	28.5	2
Fertilizin 5 minutes radiation....	21.6	Few	17.9	0	8.4	0	10.5	0	87.2	0
Fertilizin 15 minutes radiation...	17.5	0	15.6	0.5	9.5	0	13.6	0	52.1	0

results correspond closely with those previously obtained by Richards ('14) when enzymes were radiated, and suggest very strongly that sperm agglutinin may also be an enzyme.

The effect of radiation of the agglutinin wears off in two or three hours, the length of time during which eggs should be exposed to the secretion in order to produce autoparthenogenesis. If the effect on the egg activator is equally transitory, one would not expect that radiation would greatly affect its parthenogenetic power. Experiments 3 and 5, table 4, suggest, however, that the effect of radiation on the parthenogenetic power of fertilizin is similar to that on its agglutinating power.

c. The relation between concentration and agglutinating power. The work of 1914 (Richards and Woodward) also brought out

the fact that "the efficiency of the agglutinin contained in fertilizin, like pepsin (Euler, p. 132), varies with the square root of the concentration." The efficiency may be measured by the length of time the sperm remain agglutinated and the concentration by Lillie's units of strength (Lillie, '14). Curves may be plotted by letting x equal the concentration and y the efficiency. The average of twelve such curves gives a figure which corresponds very closely to the curve of the equation $y^2 = 11x$, or, $y = c\sqrt{x}$ (fig. 1). The curves are nearly identical in the ower concentrations, where experimental determination is more accu-
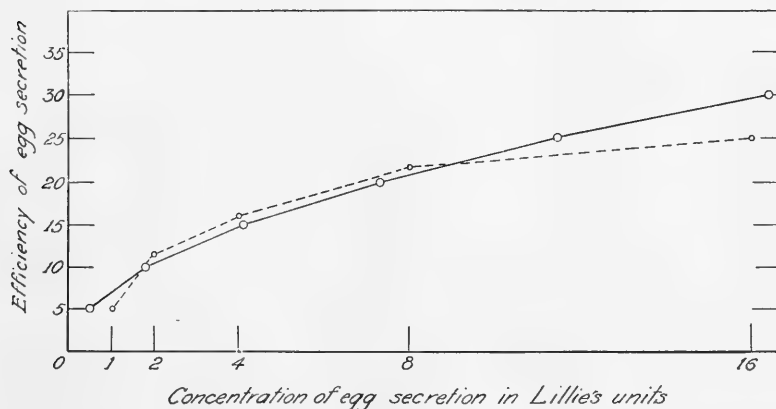
Fig. 1 The solid line is the curve of the equation $y = c\sqrt{x}$. The broken line represents the results of twelve experiments.

rate and where the greater number of values is averaged. They coincide in all parts within the limits of experimental error. It is safe to conclude, therefore, that the sperm agglutinin resembles enzymes in the relation between efficiency and concentration.

d. Tests for enzymes. If, as the radiation tests suggest, enzymes are present, it seemed likely that we might be dealing with oxidase or catalase or both. The former gives a blue reaction with guaiac, especially if hydrogen peroxide is added. If Arbacia secretion made from fresh eggs is tested with guaiac plus H_2O_2, no blue color appears. If the secretion is made from eggs which have stood for some time, so that some of them are cytolysed,

an oxidase reaction is given. It seems, therefore, that the oxidase present within the egg cannot permeate the normal membrane, and is not present in 'pure' fertilizin.

That catalase is not present in the secretion unless the eggs have been laked or cytolyzed is indicated by the fact that such an extract does not free oxygen from hydrogen peroxide (method of Loevenhart, '05). The eggs themselves, however, contain a considerable amount of this enzyme, which, like the oxidase, cannot permeate the normal membrane. This was also observed by Amberg and Winternitz ('11).

No evidence of the presence of a proteolytic enzyme could be demonstrated by the digestion of egg albumin by either fertilizin or a precipitate obtained from the secretion. This, of course, does not preclude the possibility of the presence of an enzyme that splits some Arbacia protein, but is powerless against that of a hen's egg.

While it was not expected that the secretion would contain an amylase, a mixture of fertilizin and boiled starch was allowed to stand several hours. At the end of that time, Benedict's test for sugar was applied with negative results. The experiment was also performed with a precipitate obtained from the secretion instead of fertilizin, but with negative results as before.

The only enzyme test with positive results was that for a lipase. In order to execute this test, a fatty substance was extracted from a large quantity of Arbacia eggs which had been freed from water as completely as possible by centrifuging and pipetting. The fat in these eggs was extracted with two or more volumes of ether. Later, this fatty extract, when shaken with water, formed an emulsion in which the fat globules were quite small. By measuring these in a hanging drop with a micrometer eye-piece, their diameters were found to be constant for three hours. Fresh egg secretion in which the lipase might be presumed to be present had no measurable effect on the size of the drops. It would be a mistake to conclude, however, that lipase is absent. By a modification of Robertson's method (p. 475) it is possible to throw down a precipitate from egg secretion soluble in water and capable of dissolving the oil. The

reaction with this precipitate takes place rather quickly. At
the end of the two and a half hours it was found that the de-
crease in diameter of the drops amounted to between 10 per cent
and 23.5 per cent, with an average of 16.7 per cent. This would
indicate that the secretion could change the fat into a compound
soluble in water.

3. *The precipitation of specific elements from the egg secretion
and their properties.* Of especial significance is the study of sub-
stances precipitated from egg secretions. It will be recalled
that neither Glaser nor the writer obtained a precipitate from the
egg secretion by boiling or adding absolute alcohol. Since,
however, the secretion was colloidal (not dialyzable), other
methods of precipitation were tried in 1915 and 1916.

a. Precipitation of a sperm agglutinin. If the egg secretion of
Arbacia is saturated with crystals of pure $(NH_4)_2SO_4$, a whitish
flocculent precipitate is formed. This may be washed in sat-
urated ammonium sulphate solution and dissolved in either fresh
or sea-water. The solution causes reversible agglutination of
sperm, while ammonium sulphate alone paralyzes but does not
agglutinate them. If the precipitate is dissolved in distilled
water and dialyzed in a collodian sac against distilled water or
running tap water until, as shown by testing with $BaCl_2$, all the
SO_4 is removed, it still causes reversible agglutination of sperm.
This power is lost after a few days, probably because of bacterial
action. The precipitate may, however, be kept ten days or more
without losing its agglutinative power if covered by saturated
$(NH_4)_2SO_4$, which prevents the growth of bacteria. By this
method a solution may be obtained with greater agglutinating
power than the original egg extract. The method does not,
however, bring about complete precipitation of the sperm agglu-
tinin, as was shown by dialyzing and testing the filtrate obtained
after the addition of $(NH_4)_2SO_4$. This still caused agglutination.
This precipitate has no parthenogenetic power.

b. Precipitation of a lipolysin. Since there were strong indi-
cations of the presence of lipase and perhaps other enzymes, I
adapted a method used by Robertson ('12) to obtain an enzyme
from blood serum. Eight volumes of fertilizin and four volumes

of 7 per cent $BaCl_2$ were kept at 37°C. for an hour or more and stirred frequently. The mixture was then centrifuged and the filtrate discarded. The precipitate was washed several times with $BaCl_2$ and then treated with N/10 HCl. To this solution was added Na_2SO_4 in excess to precipitate the barium, and the mixture was allowed to stand overnight. The liquid was then centrifuged and to the clear fluid were added four to five volumes of acetone, which caused a heavy flocculent precipitate. This was filtered, and the precipitate was washed several times with absolute alcohol[2] and ether, and then dried for thirty-six hours or more over H_2SO_4. The resulting powder dissolves readily in both sea-water and distilled water. It should be added that this precipitate was invariably obtained with good fertilizin and freshly distilled acetone during the summer of 1915 and until about the middle of July, 1916. It was never obtained by adding acetone to sea-water. By the middle of 1916, all of the recently purchased acetone had been used up, and recourse was necessary to some purchased from Kahlbaum in 1912. This was yellowish in color, slightly acid to litmus, and differed slightly in odor from the fresh. With this acetone, no precipitate was obtained excepting after adding NaOH, and even then only in traces.

Since (p. 474) the precipitate dissolves a fat extracted from the egg, we may call its active principle 'lipolysin.' In solution it does not agglutinate Arbacia sperm. It is, however, a very efficient parthenogenetic agent (table 5).

The solution used in these experiments contained about $\frac{1}{4}$ cc. of loose powder (about 0.025 grams) to 10 cc. sea-water. A much larger percentage of normal larvae was obtained by this precipitate than I have ever been able to obtain by any other artificial method.

It should be noted in this connection that, while the lipolysin can be precipitated from mature Asterias eggs, it has been impossible to obtain any acetone precipitate from immature ones. About 100 cc. each of A, immature, resistant starfish eggs and of

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[2] The failure to obtain a precipitate with alcohol (p. 471) was due to its dilution. While this precipitate is insoluble in absolute alcohol, it dissolves readily in 75 per cent.

B, eggs of which 50 per cent or more matured, were treated side by side, to obtain the egg extract and to precipitate it.   The precipitate from A was so slight that it produced only a faint cloudiness.   That from B, when dried, nearly filled a Syracuse watch-glass.

A sample of lipolysin prepared by this method from Arbacia eggs in August, 1915, was examined and tested in June, 1916. Its color had changed from white to a grayish violet, it had become compact, and it was much less soluble than when first made. Its efficiency as a parthenogenetic agent will be seen in table 6. Other samples of the precipitate, now a year old, do not show the physical changes noted in the 1915 sample.

TABLE 5

Lipolysin as a parthenogenetic agent

Eggs from lot	PERCENTAGE OF CLEAVAGES					
	A[1]	B	C	D	E	F
Control, unfertilized	0	0	0	0	0	0
Eggs + sperm	99	99	99	95	18	81
Eggs + hypertonic 20 minutes				0	0	0
Eggs + hypertonic 30 minutes				4	7	0
Eggs + lipolysin 2 hours + hypertonic 30 minutes				20	17	7
Eggs + lipolysin 170 minutes + hypertonic 20 minutes	31	44	53			

[1] In this and in the following tables a vertical column represents the results from the eggs of a single female or from a thoroughly mixed batch of eggs.

In $4B$ practically all the eggs which divided developed into swimming larvae.   In $6A$, 12 per cent swam; in $6B$, 10 per cent, and in $8A$ also a number swam.   It will be noted that fresh Asterias lipolysin is more efficient with Arbacia eggs than is the old Arbacia precipitate.

This parthenogenetic development was not caused by impurities, for the lipolysin does not contain a trace of barium, as proved by careful spectroscopic tests; neither does it contain acetone, alcohol, or ether, all of which were carefully removed.

Both the lipolysin and sperm agglutinin may be obtained from the same sample of egg secretion.   In order to do this, $BaCl_2$ is added first, and the precipitate saved for extraction of the lipo-

lysin. To the filtrate Na_2SO_4 is added in small quantities until all the excess of barium is precipitated. The liquid remaining can be saturated with $(NH_4)_2SO_4$ to precipitate the sperm agglutinin.

The work outlined on page 470 suggests that the egg secretion consists of at least two substances. They have now been separated, and hence we may consider the sperm agglutinin or

TABLE 6

Lipolysin not specific as parthenogenetic agent

	PERCENTAGE OF CLEAVAGES[1]	
	A	B
1. Arbacia eggs, control	0	0
2. Arbacia eggs + sperm	96	97
3. Arbacia eggs + Asterias lipolysin 1 hour	6	8
4. Arbacia eggs + Asterias lipolysin 1 hour + hyper 20 minutes	5	50
5. Arbacia eggs + Asterias lipolysin $1\frac{1}{2}$ hours	20	15
6. Arbacia eggs + Asterias lipolysin $1\frac{1}{2}$ hours + hyper 20 minutes	60	25
7. Arbacia eggs + Asterias lipolysin 2 hours	15	20
8. Arbacia eggs + Asterias lipolysin 2 hours + hyper 20 minutes	60	0
9. Arbacia eggs + Arbacia lipolysin 1 hour	10	10
10. Arbacia eggs + Arbacia lipolysin 1 hour + hyper 20 minutes	12	5
11. Arbacia eggs + Arbacia lipolysin $1\frac{1}{2}$ hours	1	0
12. Arbacia eggs + Arbacia lipolysin $1\frac{1}{2}$ hours + hyper 20 minutes	10	5
13. Arbacia eggs + Arbacia lipolysin 2 hours	25	5
14. Arbacia eggs + Arbacia lipolysin 2 hours + hyper 20 minutes	40	15

Note.—The solution of lipolysin was made by dissolving 2 cc. of the powder, shaken down in the bottom of a graduated centrifuge tube, in 15 cc. sea-water. Nos. 3 to 8, inclusive, were made with freshly precipitated Asterias powder (one week old); Nos. 9 to 14, with Arbacia precipitate ten months old.

[1] Estimated.

spermophile group, as distinct from the lipolysin, or ovophile group. The reason for Lillie's belief that fertilizin is an amboceptor is now clear. The effect of x-radiation on fertilizin and the relation between efficiency and concentration of the secretion suggest that the sperm agglutinin may be an enzyme. There is slight evidence from these tests concerning the nature of the parthenogenetic agent. When we actually apply to the precipitates tests for the individual classes of enzymes, however, it

is the parthenogenetic portion of fertilizin which suggests a positive result—one indicative of a lipase.   If the agglutinin is an enzyme, its nature is not yet known.

C. Concerning inhibitors

The dual nature of the secretion in no wise impairs the utility of inhibitors in the analysis of the fertilization reaction.   An inhibitor may function in a purely mechanical or physical manner as by making the egg membrane tough so that the sperm cannot enter, or it may remove from the reaction system one or more of the essential reagents.   Such an inhibitor, as Lillie ('14) has pointed out, may act by: 1) Removing fertilizin.   2) Occupying the 'sperm receptors.'   3) Occupying the 'spermophile side-chain' (agglutinin).   4) Occupying the 'ovophile side chain' (lipolysin).   5) Occupying the 'egg receptors.'   In other words, an inhibitor may combine with the agglutinin, the lipolysin, or the substances within the sperm or egg with which these react.   We shall consider for the present only inhibitors of organic origin.

1. Removal of fertilizin.   Repeated washing is the only method yet devised for removing fertilizin.   The discussion of the effects of this treatment, page 464, shows it to be an effective means of inhibition.

2. Combination with sperm receptors.   Godlewski ('10) found that sea-urchin eggs to which had been added Chaetopterus sperm were made resistant to sperm of their own species subsequently added.   Lillie interprets this as being due, perhaps, to a change in the spermatozoa which prevents their union with fertilizin.   In other words, he believes that from Chaetopterus sperm may be dissolved into the water some substance which combines with the sea-urchin 'sperm receptor' and occupies the group which would normally unite with the spermophile side chain of fertilizin.   The receptor so 'occupied' would be unable to combine with the fertilizin-egg complex, and therefore fertilization would not occur.   This interpretation, Lillie admits, is purely hypothetical.   No experiments to test it have been performed. It would be just as reasonable to assume that Chaetopterus sperm combined with or adsorbed the agglutinin or the lipolysin or that they in some way injured the egg itself.

3. Combination with the 'spermophile group' (agglutinin) of fertilizin. It will be recalled that (p. 460) 'anti-fertilizin' was obtained by Lillie by breaking up Arbacia eggs after most of the fertilizin had been removed through washing. He observed that a mixture of anti-fertilizin and fertilizin was unable to agglutinate sperm. The inhibition in this case he assumed to be due to occupancy of the spermophile group of the fertilizin.

4. Combination with the 'ovophile group' (lipolysin) of fertilizin. That anti-fertilizin inhibits sperm fertilization as well as agglutination, I found in experiments where the controls produced 77 per cent and 82 per cent cleavages, while the eggs treated with anti-fertilizin produced, respectively, 68 per cent and 21 per cent cleavages. It is even more efficient in blocking autoparthenogenesis in Arbacia (table 7).

TABLE 7

Inhibition of autoparthenogenesis by anti-fertilizin

	PERCENTAGE OF CLEAVAGES	
	A	B
Eggs + fertilizin $2\frac{1}{2}$ hours + hypertonic NaCl 20 minutes.........	43	57
Eggs + antifertilizin + fertilizin $2\frac{1}{2}$ hours + hypertonic 20 minutes.	10	0
Eggs + boiled anti-fertilizin + fertilizin $2\frac{1}{2}$ hours + hypertonic 20 minutes...	2	0

Since it prevents autoparthenogenesis, anti-fertilizin must combine with the parthenogenetic agent, lipolysin, or with the egg, as well as with the agglutinin. This versatility is not surprising when we remember that under the term 'anti-fertilizin' are grouped all the egg contents to which the membrane is not permeable. It must include the fatty substance with which lipolysin combines.

From the fact that this particular union with the parthenogenetic agent takes place, we should anticipate that the fatty substance itself would inhibit the development of the egg (table 8). To obtain the fatty extract, one volume of sea-urchin eggs and two or more volumes of ether were shaken and then allowed to settle into layers. The solution used consisted of 0.3 cc. of this ethereal extract diluted with 100 cc. sea-water. In experiment 3,

TABLE 8

Inhibition of cleavage by fat extracted from eggs

TREATMENT OF EGGS	PERCENTAGE OF CLEAVAGES					
	A	B	C	D	E	F
5 cc. eggs + sperm..............................	86	85	100	100	100	100
5 cc. eggs + 1 cc. 0.3 per cent ether + sperm.......	90	94	99	100	100	98
5 cc. eggs + 1 cc. 0.3 per cent ether extract + sperm..	13	82	0	29	0	0
5 cc. sperm suspension + 1 cc. 0.3 per cent ether extract + eggs......................................	19	2	13	12	3	14

table 8, 5 cc. of Arbacia eggs and sea-water were allowed to stand with 1 cc. of this solution ten minutes before the addition of the sperm. In 4, the sperm stood in the solution ten minutes before eggs were added. In both cases, fertilization was inhibited. Tests with iodine show that this extract contains an unsaturated fatty acid, probably similar to that which forms about 30 per cent of the phosphatide in Asterias eggs (Mathews, '13).

Lillie found ('14) that Arbacia blood likewise blocks sperm fertilization in the same species. My confirmatory experiments are given in table 9. The 'serum' used was obtained by filtering the perivisceral fluid, after it had stood long enough to clot. It will be noted that boiling the serum sometimes removes the inhibitor.

Since Arbacia serum does not affect the agglutinating power of fertilizin and since its ability to inhibit development of the egg can be overcome by excess of fertilizin, Lillie concluded that block in this case was produced by occupancy of the ovophile group. The following experiment shows clearly that the sperm is not affected in this block. Arbacia eggs were left standing in blood of the same species for fifteen minutes. Then they were washed

TABLE 9

Inhibition of cleavage by Arbacia serum

	PERCENTAGE OF CLEAVAGES						
	A	B	C	D	E	F	G
Arbacia eggs + sperm.........................	40	79	93	72	47	45	24
Arbacia eggs + Arbacia serum + sperm........	0	12	0	1	5	0	0
Arbacia eggs + boiled serum + sperm..........				69	1	3	43

repeatedly with sea-water and inseminated. In one case the control showed 79 per cent cleavages and the experimental eggs 12 per cent. In another case the cleavages were 40 per cent and 0, respectively. In both dishes the sperm appeared active and normal.

There seems to be no definite proof concerning the mode of action of this inhibitor. Since mammalian blood contains specific substances which prevent autolysis, we might expect echinoderm serum to contain similar substances. These should, theoretically, prevent the action of the egg enzymes and might or might not resemble the fatty inhibitor already in the egg. If they are fatty, they would probably combine with lipolysin as Lillie suggested, otherwise they might not.

TABLE 10

Inhibition of cleavage in Asterias by Asterias and Arbacia sera

	PERCENTAGE OF CLEAVAGE	
	A	B
Asterias eggs + sperm..	30	28
Asterias eggs + Asterias serum + sperm.........................	0	0
Asterias eggs + Arbacia serum + sperm..........................	0	0

The fact that Arbacia serum inhibits sperm fertilization in eggs of the same species suggested trying it with Asterias eggs, and also suggested that Asterias serum might likewise contain an inhibitor. The experiments shown in table 10 prove the suggestion correct.

5. Combination with egg receptors. 'Purple x', discovered by Glaser ('14 c), is a purple compound which appears when Arbacia sperm or egg secretion are boiled. He found that the material prevents both normal fertilization and autoparthenogenesis. Since it does not prevent the agglutination reaction of fertilizin, Glaser inferred that it did not act by combining with sperm receptors or with agglutinin. It might, however, combine with either the lipolysin or the egg. No decisive experiments have been performed as yet. Since the jelly surrounding the egg becomes swollen and sticky, sperm fertilization may be prevented

TABLE 11

Inhibition of cleavage by purple x

| | PERCENTAGE OF CLEAVAGES | | | | | | | |
	A	B	C	D	E	F	G	H
Arbacia eggs + sperm...........	Polyspermy	47	24	Polyspermy	72	73	72	84
Arbacia eggs + boiled sperm (purple) + sperm.............	4			39	27	0	0	0
Arbacia eggs + boiled sperm (colorless) + sperm..........	93	63	61	82	77	55	68	

mechanically by entrapping the sperm. The egg itself also appears swollen, and hence may be so changed that it cannot develop. It will be noted in the following experiments (table 11) that boiled Arbacia sperm blocks fertilization only when 'purple x' is present. This inhibitor (Woodward, '15) appears to be derived from testicular or ovarian tissue.

Boiling the sperm or egg secretion of Asterias produces a salmon-colored substance, 'salmon x,' which also acts as an inhibitor with both Asterias and Arbacia eggs. The color of the boiled Asterias sperm suspension was not always noted. The inconsistent results shown in the second line of table 12 arouse the suspicion that in some cases the inhibitor, and perhaps the salmon-colored compound, was not present.

TABLE 12

Inhibition of cleavage by salmon x

| TREATMENT OF EGGS | PERCENTAGE OF CLEAVAGES | | | | | |
	A	B	C	D	E	F
Asterias eggs + sperm..............................	75	14	25	37	30	28
Asterias eggs + boiled Asterias sperm + sperm.....	78	66	17	41	3	21
Asterias eggs + boiled Asterias fertilizin + sperm...	13	1	6	21		
Arbacia eggs + sperm..............................			73	72	84	
Arbacia eggs + boiled Asterias sperm + Arbacia sperm..			0	0	0	
Arbacia eggs + boiled Asterias fertilizin + Arbacia sperm..			0	0	0	

6. Unclassified inhibitors. Echinochrome, the red pigment coloring Arbacia 'blood cells,' completely inhibits the cleavage of Arbacia eggs. It is prepared by allowing Arbacia 'blood' to clot, washing the clot with sea-water to rid it of serum, and then laking the cells by adding a measured volume of distilled water. To make this solution isotonic with the eggs, it is necessary to add an equal volume of 'double sea-water,' i.e., sea-water boiled down to one-half its original volume.

It was suspected at one time that 'purple x' and echinochrome might be identical. MacMunn ('85) found that the addition of NaOH to echinochrome turned it yellowish, while HCl produced a red-yellow color. Since neither reagent produces a color change in 'purple x' (Woodward, '15), the suspicion has no foundation.

We may summarize the methods of inhibition as follows:

Foreign sperm (Godlewski, Herlant)...Which occupy sperm receptors (Lillie).
Anti-fertilizin (Lillie).................Which occupies the spermophile group (Lillie) and combines with lipolysin (Woodward).
Washing (Lillie).......................Which removes 'fertilizin' (Lillie).
Blood of same species (Lillie).........Which occupies ovophile group (Lillie).
'Purple x' (Glaser)....................Which occupies egg receptor (Glaser) or causes physicochemical change in egg (Woodward).
'Salmon x' (Glaser)....................Ditto.
Blood of related species (Woodward)..Which may combine with lipolysin (Woodward)
Fatty extract from eggs (Woodward)...Which combines with lipolysin (Woodward).
Echinochrome (Woodward)............Which appears to change egg itself (Woodward).

III. CONCERNING ACTIVATORS—PARTICULARLY LIPOLYSIN—AND THEORIES OF ACTIVATION

To the eye, 'activation' of an egg is manifested by cleavage. Side by side with this goes an acceleration of metabolism indicated by a three- to five-fold increase in the rate of oxidation in developing over resting eggs (Warburg, '08 and Loeb and Wasteneys, '13). This increased metabolism is also found in cytolyzing eggs.

A. *Artificial activation*

In our attempts to understand activation, the method of imitating the effect of sperm by physical and chemical means has been of the greatest service. The first change to be noticed when an echinoderm egg is fertilized is the appearance of a 'fertilization membrane.' Such membrane formation may be brought about by a large number of agents (Loeb, '13, '16), distilled water, the lower fatty acids, lipoid solvents, bases, certain salts, increased temperature, shaking in some cases, saponin, solanin, and bile salts. All of these cause cytolysis. If the cytolysis is confined to the cortical layer of the egg, a membrane will be formed and the egg will develop. Most of these agents, however, if left to themselves, cytolyse not only the cortical layer, but the whole egg. Loeb found it necessary, therefore, either to follow the cytolytic agent with treatment by hypertonic solutions in the presence of oxygen or to inhibit oxidation. The latter may be accomplished by depriving the eggs of oxygen or by treating them with a cyanide.

So far as is known, there are only three classes of physiological activators, the egg secretion of the same or related species, the blood-serum of unrelated species, and, finally, the spermatozoön. That the egg secretion may cause superficial cytolysis is indicated by the work of Glaser, who found that it increases the permeability of Arenicola larvae. Many investigators, notably Friedenthal, have found that mammalian blood-serum contains something which cytolyses cells of unrelated animals. Hence it is not surprising to find that foreign blood and extracts of foreign cells cause cytolysis and activation of Echinoderm eggs (Loeb, '13).

On the basis of these facts, Loeb ('12) concludes that "the spermatozoön, as well as the blood and tissues, contains a substance (lysin) which causes only cytolysis of the cortical layer." Such a theory brings the phenomena of parthenogenesis and sperm fertilization into one class.

F. R. Lillie, on the other hand, believes that the spermatozoon "functions essentially as the activator of a third body, 'ferti-

lizin,'" which, in turn, causes cleavage. Such an activation of fertilizin might be conceived as an increase in the affinity of the ovophile group for the egg receptor (Glaser). This hypothesis seems difficult to confirm. How are we to get rid of the sperm in a sperm-fertilizin mixture without injuring the fertilizin? Sperm pass readily through any available filter through which fertilizin can pass, but can be killed by heating to 40°C. for three minutes. Since fertilizin does not lose its ability to agglutinate sperm if boiled for a few minutes, a method suggests itself.[3]

Equal amounts of Arbacia fertilizin were put into two test-tubes and to one was added a large amount of Arbacia sperm. They were allowed to stand fifteen minutes, then both tubes were

TABLE 13

A comparison of the parthenogenetic effects of fertilizin with those of fertilizin 'activated' by sperm

	PER CENT CLEAVAGES
1. Eggs (Control 1)...	0
2. Eggs + fresh sperm (control 2)....................................	70.5
3. Eggs + heated fertilizin (diluted to $\frac{1}{2}$) 2 hours + hypertonic 20 min.	61
4. Eggs + heated fertilizin (diluted to $\frac{1}{2}$) 2 hours.....................	22
5. Eggs + heated mixture of fertilizin and sperm (diluted to $\frac{1}{2}$) 2 hours	21
6. Eggs + heated fertilizin (diluted to $\frac{1}{4}$) 2 hours.....................	0
7. Eggs + heated mixture of fertilizin and sperm (diluted to $\frac{1}{4}$) 2 hours	0

heated in a water-bath to 40°C. for three minutes. Eggs were then treated as in table 13. In experiment 4 and those following, hypertonic after-treatment was not given, since that in itself causes development in some cases, and I wished to learn the effect of the 'activated fertilizin.' Dilutions were used because it was thought that they might show more clearly the greater activity of fertilizin mixed with sperm. Repetition of this experiment gave essentially the same results.

Since the heated fertilizin alone caused development in 22 per cent of the eggs and that to which sperm had been added caused

[3] Glaser first attempted to solve this problem, but failed because he boiled the mixture of sperm and fertilizin, and thus obtained 'purple x,' which inhibited development.

cleavage in 21 per cent, the indication is that the sperm does not activate the fertilizin.   Of course, this is not conclusive, since the temperature which kills the sperm may also destroy the 'activated' fertilizin, and unless this is broken down into the un-activated form, we should expect fertilizin treated with sperm to be markedly weakened.   Since this is not the case, we shall have to conclude that an activating effect on the ovophile group, or lipolysin, is undemonstrable.   But why should we expect activation?   The 'ovophile' and 'spermophile' groups are two independent substances.   Such being the case, it is not surprising that a reaction involving one does not 'activate' the other.

Since Lillie's idea of activation cannot be accepted, we are brought back to superficial cytolysis and its connection with the activation of the egg.   It is quite natural that superficial cytol-ysis should have been considered a cause of activation.   On this assumption, the search for a modus operandi has been carried on by several investigators.   Loeb himself suggests that cytol-ysis removes an obstacle to development.   R. S. Lillie ('11) be-lieved the obstacle might be CO_2.   Loeb proved that this was impossible by using sea-water charged with CO_2 as a partheno-genetic agent.   Glaser suggested that the obstacle might be anti-fertilizin.   Both R. S. Lillie and Glaser postulated that the removal of these obstacles was due to the increased permeability of the egg resulting from cytolysis.

But suppose that cytolysis is not the cause of activation, but an effect (of minor significance) of other processes which produce development?   From this standpoint we get a different view of the situation.

The egg has been found to contain a considerable amount of lipoid, about 30 per cent of which is an unsaturated fatty acid. Overton and others have shown that this lipoid must be more concentrated at the surface than in the interior.   According to Jobling, an unsaturated fatty acid inhibits enzyme action. We know that the egg contains a quantity of enzymes—catalase and oxidase, for instance—which control metabolism.   Let us assume that the activity of the enzyme is decreased by the pres-ence of the unsaturated fatty acid.   Removal of this fatty acid,

then, should result in increased metabolism. With this assumption in mind, let us examine the methods of artificial parthenogenesis.

Lipoid solvents, such as butyric acid, chloroform, chloral hydrate, and ether, can remove the fatty inhibitor by dissolving it, and thus allow the enzymes to act more rapidly. Bases saponify fat. The third large class of chemicals used for this purpose includes the halogen salts. These are usually used in hypertonic solutions, but still sufficiently dilute so that there is considerable dissociation. The basic radical, as before, can saponify the fat. But the halogen radical is the more important, for that can saturate the acid, which no longer inhibits enzyme action after saturation (Jobling and Petersen). The compounds of bromine and iodine are more effective than the chlorides as parthenogenetic agents, just as they more easily saturate fatty acids. Such a solution must be hypertonic to allow the salt to penetrate a membrane tuned to normal sea-water. Finally it must not be forgotten that lipolysin is both a fat solvent and a parthenogenetic agent.

That these parthenogenetic agents act by removing an inhibitor is indicated by experiments of R. S. Lillie ('12), who found that 'resistant' Asterias eggs at the close of the season would give a much greater percentage of cleavages if treated with a very dilute solution of lipoid solvent or halogen compound before fertilization with sperm.

Early in June, 1915, I found few Asterias eggs mature, and those in which the germinal vesicle had broken down failed to attract sperm in any visible numbers. Some of these eggs were treated with solutions of lipoid solvents and other compounds (tables 14 and 15). In the treated eggs, mature ova and cleavages were subsequently found in much larger percentages than in the controls. The treatment not only enabled the sperm to fertilize more mature eggs, but enabled more eggs to form polar bodies.

Sperm to which had been added resistant Asterias eggs were neither more nor less active than before and did not collect around the eggs. They became very active, however, if the eggs had

been previously treated for one hour with 0.3 per cent solution of ether in sea-water and then washed thoroughly. Moreover, from four out of eleven females tested, the treated eggs, whether mature or not, became surrounded by dense halos of sperm (see

TABLE 14

The effect of lipoid solvents on resistant Asterias eggs

	A		B[1]		C		D		E		F		G		H	
	Control	Inseminated	Control	Inseminated	Control	Inseminated	Control	Inseminated	Control	Inseminated	Control	Inseminated	Control	Inseminated	Control	Inseminated
Untreated controls...............		9		8	1	1	0	1	0	1	0	0	0	2	0	9
Ether, 0.3 per cent																
60 minutes...................	0	46	0	50	2	56	0	37	0	17	0	20	0	15	0	19
90 minutes...................	0	44	0	50												
Chloroform, saturated																
35 minutes...................	0	53	0	50												
48 minutes...................	0	46	0	50												
Chloroform, one-fourth saturated																
45 minutes...................	0	39	0	40												
60 minutes...................	0	26	0	40												
75 minutes...................	0	20	0	20												
Chloretone, 0.1 per cent																
35 minutes...................	0	48	0	25												
55 minutes...................	0	36	0	25												
90 minutes...................	0	25	0	25												
Chloral hydrate, 0.1 per cent																
50 minutes...................	0	54	0	55												
2 hours......................	0	44	0	50												
3 hours......................	0	7	0	Few												
Butyric acid, 0.5 cc. $\frac{N}{10}$ + 50 cc. sea-water																
12 minutes...................	0	45	0	50												
20 minutes...................	0	48	0	50												
40 minutes...................	0	46	0	50												

[1] Estimated.

photographs). The others were unaffected. When, late in the season, Asterias eggs were again resistant, this experiment was repeated. Out of ten batches of eggs treated, every one attracted sperm decidedly better than the control.

Since the inhibitor present in the egg is a compound of an unsaturated fatty acid, iodine should saturate the acid and enable the egg to develop. The validity of this reasoning is shown by experiments in which Miss Hague and the writer ('17) used iodine as a parthenogenetic agent with Arbacia eggs. The curves in fig. 2 and the data in table 16 summarize the results by

TABLE 15

The effect of salt solutions on resistant Asterias eggs. The amount of reagent indicated in the first column was added to 100 cc. sea-water. The eggs were treated with these solutions for twenty minutes, washed, and inseminated

| | PERCENTAGE OF CLEAVAGES | | | | | |
| | A | | B | | C | D |
	Control	Insemi-nated	Control	Insemi-nated	Insemi-nated	Insemi-nated
Untreated controls........	0	4	1	11	5	25
Calcium chloride						
4 cc. 2.5 molecular.....	20	24			22	
2 cc. 2.5 molecular.....	10	38			15	
1 cc. 2.5 molecular.....			1	7		18
5 cc. 2.5 molecular.....			1	7		9
Lithium chloride						
8 cc. normal..........	6	36			16	
4 cc. normal..........	3	53			19	
2 cc. normal..........			1	9		31
1 cc. normal..........			1	8		25
Potassium iodide						
4 cc. normal..........	3	48			26	
2 cc. normal..........	1	47			23	
1 cc. normal..........			1	10		25
0.5 cc. normal........			1	10		15

Note.—The addition of very dilute KCNS (about 1 cc. of a 1 per cent solution to 100 cc. of sea-water) likewise produced a great increase in the number of eggs fertilized.

giving the average number of cleavages obtained by each method of treatment. The fact that the length of time during which the eggs are subjected to iodine does not affect the number of cleavages, indicates that the reagent not only enters the egg immediately, but also affects it immediately to its fullest extent. This strengthens the idea that iodine accomplishes its result by combining with the unsaturated fatty inhibitor. The membranes
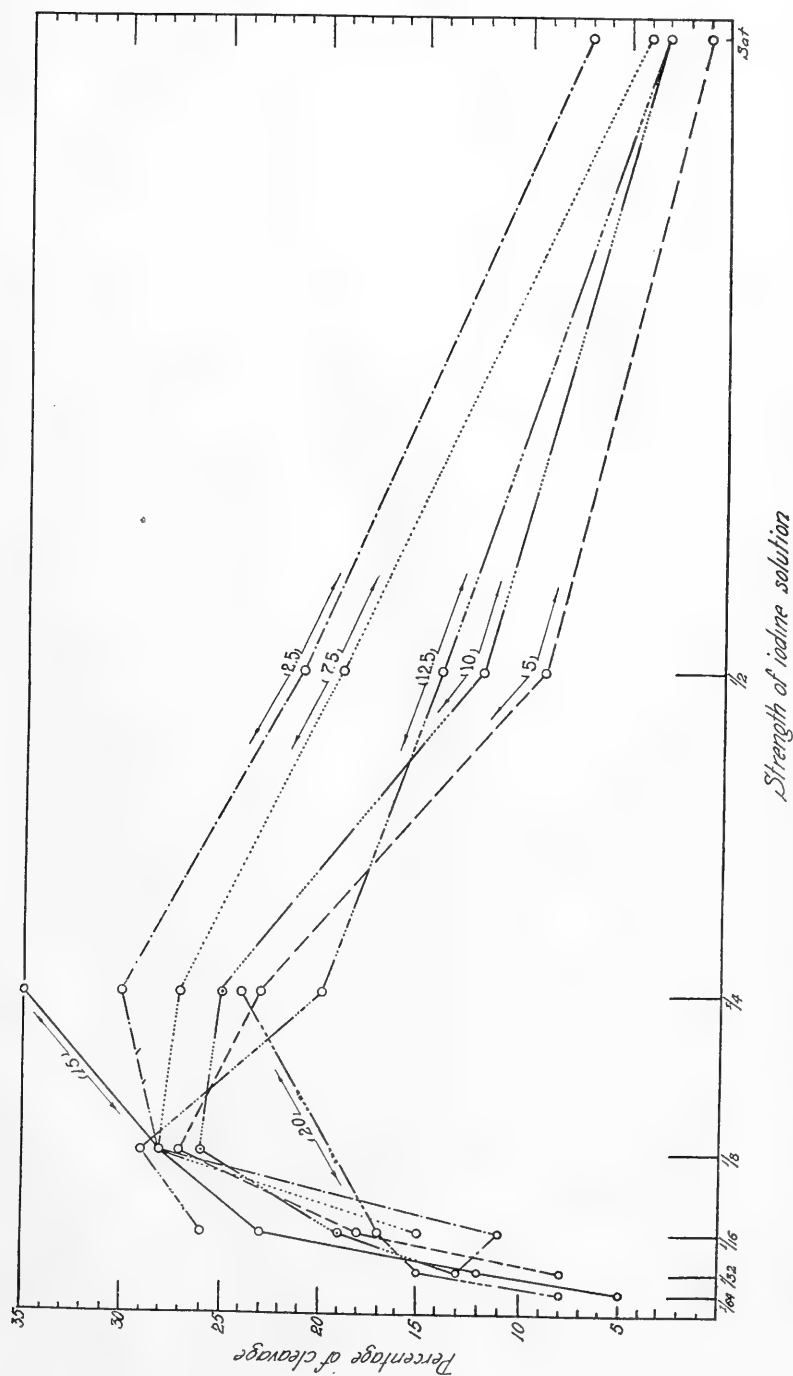
Fig. 2 Curves showing the percentage of changes obtained by using iodine as a parthenogenetic agent. The numbers on the curves indicate the number of minutes during which the eggs were exposed to iodine.

formed on these eggs were remarkable in resembling the membranes on eggs fertilized with sperm more closely than those on the usual parthenogenetic eggs—another reason for the belief that iodine reacts with the normal inhibitor and so permits the normal activator to function.

Arbacia eggs, like those of Asterias, become resistant to fertilization toward the close of the breeding season. Their fertility may likewise be increased by allowing them to stand in dilute iodine solution ten minutes before insemination. It should be noted that iodine treatment, like any other form of 'doctoring,'

TABLE 16

The parthenogenetic effect of iodine

TIME	SATURATED IODINE SOLUTION		$\frac{1}{2}$ SATU-RATED IODINE SOLUTION		$\frac{1}{4}$ SATA-RATED IODINE SOLUTION		$\frac{1}{8}$ SATU-RATED IODINE SOLUTION		$\frac{1}{16}$ SATU-RATED IODINE SOLUTION		$\frac{1}{32}$ SATU-RATED IODINE SOLUTION		$\frac{1}{64}$ SATU-RATED IODINE SOLUTION		CONTROLS	
		+Hypertonic		+Hypertonic		+Hypertonic		+Hypertonic		+Hypertonic		+Hypertonic		+Hypertonic		+Hypertonic
minutes																
2.5	2	7	15	21	21	30	14	28	9	11	8	13			4	6
5.0	1	1	15	9	22	23	19	27	11	18	6	8			3	5
7.5	7	4	18	19	29	27	26	28	6	15					2	5
10.0	3	3	6	12	24	25	20	26	14	19	4	13			3	6
12.5	11	3	9	14	18	20	16	29	16	26					3	4
15.0					23	35	17	28	15	23	10	12	2	5	3	5
20.0					21	24	9	5	15	17	14	15	2	8	4	5
25.0									15	20	8	16			7	9

does not improve eggs that are already fairly normal, like those in the last column of table 17.

In this connection it is interesting to recall the experiment of E. P. Lyon and L. F. Shackell ('10). They found that iodine, as indicated by the starch reaction, disappeared more rapidly from sea-water in the presence of unfertilized eggs than of eggs which had been fertilized. From this they concluded that the unfertilized eggs are more permeable to iodine than the fertilized. In the light of my own experiments, it seems more probable that the unfertilized eggs contained more unsaturated fatty acid with which the iodine might combine.

On the other hand, if the eggs be allowed to settle in test-tubes and only the supernatant fluid be tested, it will be found that the liquid above the unfertilized eggs combines less readily with iodine than does that above the fertilized eggs. The first inference from this was that the fertilized eggs had excreted unsaturated fatty acid into the sea-water. If, however, we add to the water poured off from unfertilized eggs an amount of sperm suspension equal to that present in the other tube, we find that iodine is absorbed in equal amounts from each. Therefore, the results were due to the presence of sperm in the water above fertilized eggs, rather than to unsaturated acid excreted at the time of fertilization.

TABLE 17

The effect of iodine on resistant or overripe Arbacia eggs

		PER CENT		
1.	10 cc. eggs + sperm, control.............................	24	29	81
2.	10 cc. eggs + 1 drop saturated iodine 10 minutes + sperm...	28	39	40
3.	10 cc. eggs + 2 drops $\frac{1}{4}$ saturated iodine 10 minutes + sperm...	36	44	62
4.	10 cc. eggs + 1 drop $\frac{1}{4}$ saturated iodine 10 minutes + sperm...	50	48	61
5.	10 cc. eggs + 1 drop $\frac{1}{8}$ saturated iodine 10 minutes + sperm...		31	

Neither Asterias nor Arbacia lipolysin seems to combine with iodine. The chemical parthenogenetic agents, however, react with the fatty inhibitor, and, as we saw, the egg secretion, one of the three physiological activators, also combines with it to produce a compound soluble in water.

According to this view, then, the resting egg contains enzymes which control metabolism, unsaturated fatty acid which inhibits enzyme action, and lipolysin, which reacts with the unsaturated fatty acid to make it innocuous. We can conceive that the relative amounts of these substances may change from time to time in the same egg and may differ in different eggs. If there is more than sufficient fatty acid present to combine with all the lipolysin, the surplus will inhibit the action of the enzymes and the egg

will lie dormant. As the egg 'ripens,' however, it secretes more and more lipolysin. If this accumulates within the egg to such an extent that the inhibitor is relatively greatly reduced, the metabolic enzymes become extremely active and development or cytolysis results. Perhaps natural parthenogenesis, such as occurs in Daphnia and aphids is the result of such conditions. The membrane of starfish and sea-urchin eggs is easily permeable to lipolysin, so that it does not normally accumulate within the egg. If, however, the eggs are crowded in a small amount of sea-water or, which amounts to the same thing, are placed in a bath of lipolysin, the loss of this substance from the egg is prevented. It therefore reacts with the inhibitor, and the egg develops—a case of autoparthenogenesis.

Any other method which disturbs the ratio of activating enzymes to fatty acid so that the proportion or effectiveness of the enzymes is increased must lead to development or cytolysis. As already stated, many chemicals have this effect, and produce parthenogenesis.

B. Activation by sperm

Can the action of the spermatozoön be explained along similar lines? Its effect probably varies with the group of animals. Ostwald ('07) measured the amount of peroxidase and catalase in 1) extracts of Amphibian eggs; 2) extracts of sperm; 3) a mixture of the two extracts. He found that the mixture contained considerably more enzyme than the sum of (1) and (2). Hence we may conclude that in Amphibia the sperm carries into the egg something which changes pro-enzymes into enzymes. It increases the activator. On the other hand, Amberg and Winternitz ('11) were unable to find any more oxidase or catalase in echinoderm eggs laked after fertilization than in those laked before. Moreover, Warburg ('08) and Loeb and Wasteneys ('13) found the same rate of oxidation in parthenogenetic as in fertilized sea-urchin eggs. Since, in this group, fertilization does not increase the amount of oxidase in the egg, the spermatozoön may act by reducing the amount or effect of the inhibitor.

In conclusion, I wish to express my gratitude to Dr. O. C. Glaser, who suggested this problem and directed the work; to Dr. F. R. Lillie for the privileges of the Marine Biological Laboratory, Woods Hole, and to the several colleagues who kindly allowed me to demonstrate to them various reactions.

IV. SUMMARY

In confirmation of the work of Lillie and Glaser, it was found that Asterias and Arbacia eggs secrete into the supernatant seawater a substance which causes the sperm of the same species to be activated, aggregated, reversibly agglutinated, and paralyzed. The secretion is also a parthenogenetic agent.

Further study of its physiological properties brought out:

1. That its presence is necessary for the fertilization of the egg, since,

   a. Immature eggs of Asterias, which cannot be fertilized, produce a secretion with less than one-sixtieth the agglutinating power of that produced by the same eggs when mature.

   b. Eggs from which the secretion has been washed do not develop when inseminated. If, however, secretion be added before insemination, they develop.

   c. Arbacia eggs which are 'resistant' to fertilization late in the season, also produce little secretion. They fertilize normally if secretion is added.

2. That the secretion has a dual nature, as shown by the following facts:

   a. It reacts with both the sperm and the egg.

   b. Boiling destroys its value as a parthenogenetic agent, but not as an agglutinin.

   c. Perivisceral fluid of the same species inhibits autoparthenogenesis, but not agglutination.

A qualitative study of the chemical properties of the secretion confirmed Glaser's observations, and indicated:

1. That it does not dialyse through a collodion sac, and so is probably colloidal.

2. That it contains carbon and nitrogen, but gives no clear response to protein tests. It gives a faint yellow color, in the

xanthoproteic test, however, which indicates the presence of tyrosine, phenylalanine, or tryptophane.

3. That two substances can be precipitated from the same secretion:

  a. By saturation with $(NH_4)_2SO_4$, a sperm agglutinin is thrown down.

  b. By a method of Robertson and others, using $BaCl_2$ and acetone, a parthenogenetic agent is obtained.

In an attempt to learn whether or not enzymes are present, it was found that the agglutinin resembles an enzyme in the effect upon it of x-radiation. It also follows the law of Schütz and Borissow, i.e., rate of action $= K\sqrt{C}$ ferment. The secretion does not give a positive reaction to tests for oxidase, catalase, or a proteolytic enzyme. Since the parthenogenetic agent dissolves a fat obtained from the eggs, it may contain a lipase. Hence it was called, provisionally, a lipolysin.

A study of methods of inhibiting the initiation of cleavage brought out that both autoparthenogenesis and sperm fertilization are inhibited by:

  1. Asterias and Arbacia serum, which may, like mammalian sera contain antiferments specifically protecting the cells of the organism itself.

  2. 'Purple x' and 'Salmon x,' substances obtained by boiling the testicular or ovarian tissues of Arbacia and Asterias.

  3. Antifertilizin, obtained from eggs which have been freed from fertilizin. This inhibitor may be identical with

  4. An unsaturated fatty acid obtained by extracting the eggs with ether.

Parthenogenetic agents fall into three classes: 1) The fat solvents, including lipolysin, ether, chloroform, butyric acid, etc. 2) The halogen compounds—iodine and various salts. 3) Physical methods, which may change the physical or quantitative relations of substances within the egg. Not only do these agents produce development in normal, uninseminated eggs, but they change resistant Asterias and Arbacia eggs so that they may be fertilized.

A consideration of the above facts makes it seem probable that the factors tending to produce development in the resting egg are of the nature of enzymes. The action of these, Jobling found, may be inhibited by unsaturated fatty acids. The egg remains in the resting stage so long as the action of these enzymes is inhibited by the unsaturated fatty acid. The egg itself, when mature and in a suitable medium, produces a lipolysin which binds this inhibitor. The efficiency of the inhibitor may also be lowered by physical and chemical means. In some groups, the spermatozoon appears to bind the inhibitor, in others, to increase the activity of the enzymes.

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Photomicrographs of inseminated Asterias eggs:

1 and 2   Controls. Note that the spermatozoa are scattered. This shows especially well in the right hand part of 1.

3   Eggs from the same female treated for one hour with 0.3 per cent ether in sea-water, washed, and then inseminated, with the same sperm suspension as above.   Note the spermatozoa in halos around both mature and immature eggs.

1

2

3
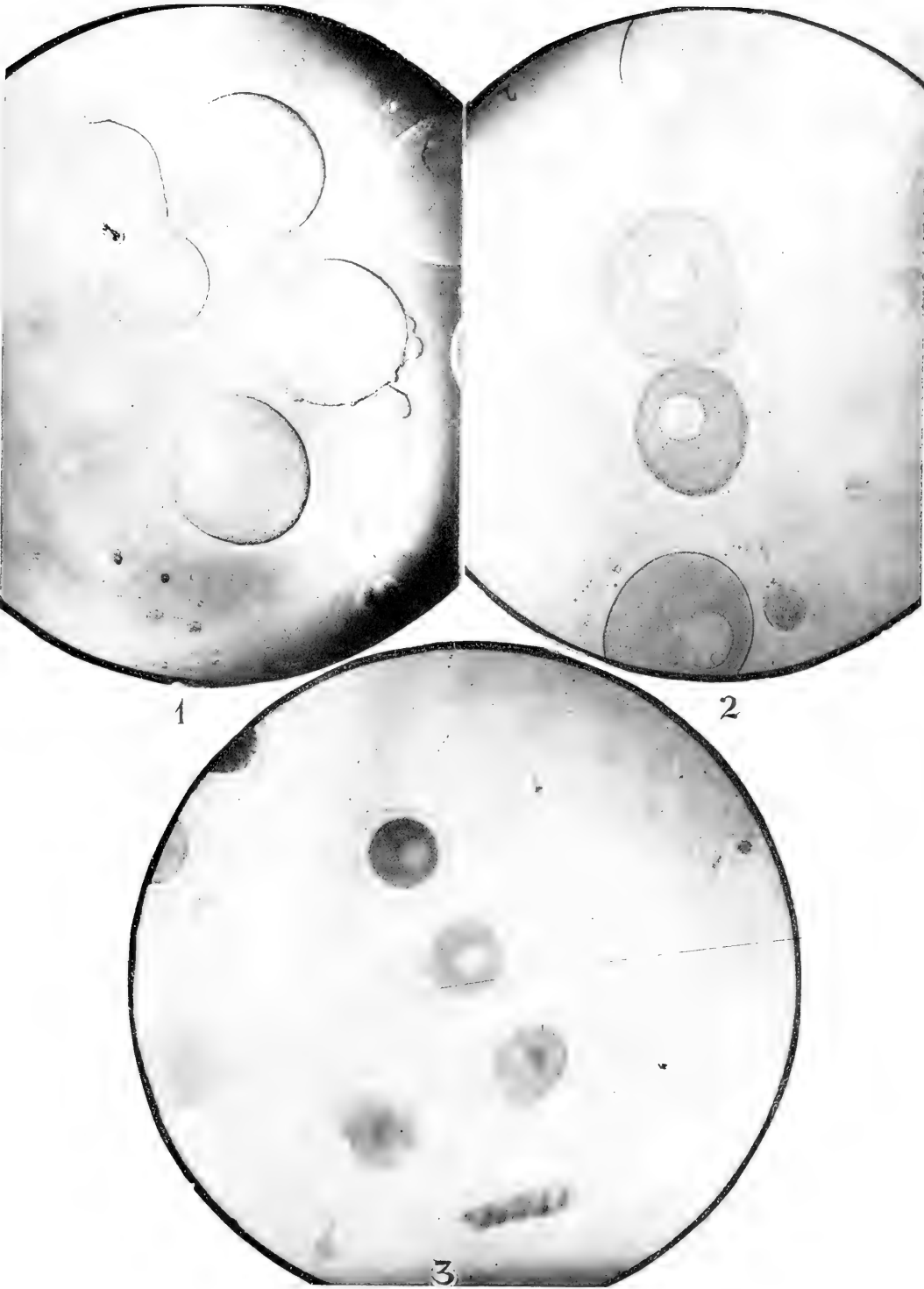
EFFECTS OF CHEMICALS ON REVERSION IN ORIENTATION TO LIGHT IN THE COLONIAL FORM, SPONDYLOMORUM QUATERNARIUM

S. O. MAST

Zoological Laboratory of the Johns Hopkins University

CONTENTS

INTRODUCTION

The problems in behavior may be divided into two groups, one containing those which concern the nature and the cause of responses, the other those which concern the cause of changes in responses. The problems in the former group deal extensively with the relations between the responses and the environment or certain processes within the organism; those in the latter deal primarily with habit formation or learning, regulation or adaptation and evolution, i.e., with some of the most fundamental characteristics of living matter.

The investigations which have thus far been made in behavior belong largely to the former group. Many observations have, however, also been made on changes in the responses. In fact, from a qualitative point of view, the whole field has been surveyed

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to such an extent that, while doubtless many more facts of a qualitative nature will be discovered, this is not likely to result in the formulation of principles of much importance.   Advance in behavior undoubtedly depends largely upon intensive quantitative research.

This paper deals with the beginning of a series of quantitative investigations concerning changes in the nature of responses. Reversion in the sense of orientation was selected for this study because it appears to be among the simplest of the changes in responses, and if it occurs at all it is always fairly complete and fairly well defined, so that it serves well for work requiring precise measurements.   The selection of an organism that can be cultivated in the laboratory is also of importance, for in organisms that thrive in the laboratory normal behavior can be much more readily ascertained than it can in those which do not.   Thus difficulties such as Esterly ('17), for example, encountered in work on the marine copepods are readily avoided.

At present we are interested primarily only in reversal in orientation in light.   The literature on this subject has recently been fairly thoroughly reviewed.   (Mast, '11, pp. 265–287; Holmes, '16, pp. 93–119; Washburn, '17, pp. 200–208).   Reference to these reviews and to a few investigations not mentioned in them leads to the following conclusions:

1. Organisms are usually positive in weak and negative in strong illuminations.   Holmes ('01, · '05), however, maintains that the opposite holds for Orchestia and Ranatra, and there are many organisms in which reversion cannot be induced by light.

2. Reversal in the sense of orientation in light is probably usually dependent upon the amount of light energy received. This has been fairly clearly demonstrated by Arisz ('15) for plants and Mast ('07, pp. 154–162) for Volvox.   Under certain conditions, however, reversion may depend upon the time-rate of change in light intensity.   Gamble and Keeble ('03, p. 397) maintain that sudden increase in illumination causes positive Convoluta to become temporarily negative.   Ostwald ('07) contends that the same reversion is produced in Daphnia by either a sudden increase or a sudden decrease in illumination.   Ewald

('14), on the other hand, holds that a sudden decrease makes Daphnia positive and a sudden increase negative.

3. Increase in temperature generally makes organisms positive to light and decrease usually makes them negative. But Loeb ('05, p. 276) maintains the opposite holds for Polygordius larvae and Holmes ('05) comes to the same conclusion in reference to Ranatra; moreover, in some organisms temperature appears to have no effect on reversion.

4. Nearly all of the observations on the effect of chemicals on reversion in orientation have been made on crustacea (larvae and adults) and on Arenicola and Balanus larvae. None of the unicellular forms have been tested in respect to this and only one colonial form, Volvox, has been studied, and in this one only the effect of acids. These observations lead to the following conclusions:

Acids tend to make all of the forms studied positive with the exception of Arenicola, but in many instances some of the salts, alkalis, and narcotics have the same effect as the acids. It is thus evident that chemicals which are fundamentally different in properties may have the same effect on reversion. Consequently reversion in the crustacea cannot be specifically related to the chemical constitution of the environment. It is therefore probably related to the physiological state of the organisms as a whole, but as to the nature of the physiological states involved in reversion we are as yet in total darkness. The interesting experiments of Allee ('18, p. 95) show that in the May-fly nymphs it is not specifically associated with stimulation or depression. Allee found that hydrochloric acid and ethyl alcohol cause reversion from negative to positive, but that in some cases the nymphs were stimulated and in others depressed as indicated by the rate of production of carbon dioxid. Thus it is evident that in these relatively complex forms the problem is greatly involved. In the simpler forms it is not unreasonable to expect a more direct relation between the environmental factors and reversion.

In a few of the investigations on the effect of chemicals on reversion solutions of known concentration were used, but in most of them the chemicals to be tested were merely added in indefinite

amounts to the solutions in which the organisms live. Under such conditions it is, of course, impossible to say precisely what the chemical constitution is and how it is affected by the addition of chemicals. For example, most fresh waters are distinctly alkaline. When acid is added to such a solution it is evident that it can affect organisms as acid only after. the alkalis are neutralized. Addition of acids may, therefore, merely subject the organisms to an increase in salts and a decrease in alkalinity. The importance of this will become evident as we proceed in our discussion.

MATERIAL AND METHODS

Spondylomorum is a colonial organism consisting of sixteen zooids. It is ellipsoidal in form, about .05 mm. long and .035 mm. wide. Each zooid contains among other structures a prominent eye-spot and two flagella, considerably longer than the colony. The colonies are fairly active. They are definitely postero-anteriorly differentiated and always swim with the anterior end or surface ahead, rotating continuously on the longitudinal axis. They respond definitely to light and orient fairly precisely, being positive under certain conditions and negative under others.

Spondylomorum is not very common. It is usually found in stagnant pools rich in decaying organic matter. In October, 1912, it was found in great abundance by my colleague, Prof. E. A. Andrews, in a small puddle near a well frequented by ducks and chickens. I am greatly indebted to him for supplying me with numerous collections of this material. In the laboratory I succeeded for several months in raising the colonies in hay infusion, and in some cultures they became very abundant, but in January they all died out. I had intended to continue the work on these forms and delayed publication, hoping to obtain more laboratory cultures, but in this I have been unsuccessful.

Two methods were used in ascertaining the effect of different chemicals on the sense of orientation in light: 1) Numerous colonies were put into about 1 cc. of solution from the culture jar or pure distilled water in a square watch-glass. This was then placed at a given distance from a window and left until the col-

onies had collected either at the window side or at the room side of the dish, depending upon whether they were positive or negative; then traces of the chemical to be tested were successively added, the solution being thoroughly stirred after each addition, until the sense of orientation changed or until there was no longer any orientation. The solutions were then tested for alkali with neutral red, and for acid with salts of neutral red prepared by treating neutral red with ammonium hydrate and washing the crystals produced in pure distilled water.   2) Ten watch-glasses containing a given amount of solution from the culture jar without any colonies were placed in the same illumination in front of a window. Chemicals to be tested were then added to the watch-glasses in such amounts as to make a series of solutions differing in concentration. A drop of solution from the culture jar containing colonies was then added to the solution in each watch-glass and the effect on the behavior of the colonies noted. In all cases the solutions were tested with neutral red. In some experiments distilled water was used in place of the solution from the culture jar and the colonies were washed in distilled water before they were used.

In each experiment the temperature and illumination were practically constant throughout.

EFFECT OF CHEMICALS, GENERAL STATEMENT

The results obtained in reference to the general effect of different chemicals when added to culture solutions may be summarized as follows:

All the acids tested (carbonic, hydrochloric, nitric, sulphuric, formic, boric, chromic, tannic, tartaric, and oxalic), chloroform, ether, and chloral hydrate cause negative specimens to become strongly positive. They have no effect on positive specimens except perhaps to make them more strongly positive. There is also some evidence indicating that ethyl alcohol, ammonium chlorid and pure water induce reversion from negative to positive orientation, but if these substances actually have any effect it certainly is far less pronounced than that produced by any of the substances mentioned in the preceding paragraph.

Formalin, sugar, oxygen, hydrogen peroxid, all of the alkalis tested (NaOH, KOH, NH$_4$OH) and all of the salts tested except ammonium chlorid (MgSO$_4$, NaCl, CaCl$_2$, KNO$_3$) have no appreciable effect on the sense of orientation.

These results, as presented, throw but little light on the question as to the factors involved in reversion. Let us, therefore, consider in detail the effects of the substances mentioned.

EFFECT OF ACIDS

If a little acid is added to a solution containing negative colonies of Spondylomorum, they usually become strongly positive almost immediately, but they soon become negative again. If now more acid is added, they become positive again, but in a few moments they are again negative. Thus they continue to become positive after every addition of acid and then negative again until they are killed. These features in the response of Spondylomorum are clearly brought out in the following details regarding two experiments:

1. On November 27, at 2.40 p.m., numerous colonies were put into a watch-glass containing about 1 cc. of solution from the culture jar and exposed in diffuse daylight. The colonies immediately collected at the side of the watch-glass away from the source of light. They were strongly negative. At 2.51 p.m., a trace of 10 per cent sulfuric acid was added, the solution being thoroughly stirred at the same time. At 2.53 p.m., the colonies were clearly slightly positive; but at 2.55 p.m., they were beginning to become negative, and at 3.00 p.m., they were strongly negative again. A trace of acid was now added, after which the colonies immediately became fairly strongly positive; but at 3.03 p.m., they were again negative. More acid was added at this time, and the colonies again immediately became distinctly positive. At 3.06 p.m., they were again negative. Acid was again added, and reversion followed immediately with a return to negative orientation at 3.10 p.m., when the process was again repeated with the same results. A trace more acid was now added. The colonies became inactive, but in a few moments they became active again and swam definitely toward the light,

but three minutes later they were again negative. A minute trace of acid was again added, the colonies became inactive and all died in a few moments.

2. On December 6 sodium hydrate was added step by step to a solution containing numerous Spondylomorum colonies, until enough had been added to make the solution n/62 NaOH on the basis of pure water. It gave a faint alkaline test with litmus-paper. In the beginning of the experiment some of the colonies were positive, others were negative. At the close all were neutral, but no reversion in orientation had occurred. The following day the colonies in the alkaline solution were in excellent condition, and five days later, December 12, they were still apparently normal and strongly negative. At this time n/100 HCl was slowly added step by step. The solution was tested with neutral red from time to time and the orientation was noted. The results obtained are presented in tabular form in table 1.

The results obtained in these two experiments are essentially like those obtained in all of the numerous other similar tests made. They show clearly that the addition of acids causes negative Spondylomorum to become positive, but they also seem to show that the reversion in orientation produced by the addition of acids is not due to the direct effect of the acids on the organisms, for the colonies in most cases became positive before the alkalinity of the culture solution was neutralized. Moreover, the results obtained with pure distilled water support this contention.

In distilled water of high[1] purity Spondylomorum lives for days and responds normally. In such solution the addition of a very small amount of acid proves fatal. For example, in one experiment, specimens put into n/15,000 HCl lived only a few moments. In n/6666 HCl they lived for some time, and in n/20,000 HCl they were still alive and apparently normal after two days. Whether or not addition of acid to pure distilled water causes reversion in orientation was not definitely ascertained,

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[1] The water used in these experiments was very generously supplied by the late Prof. H. C. Jones. It was redistilled from two Jena glass flasks in series, one containing chromic acid, the other barium hydrate, and condensed in a block-tin condenser.

TABLE 1

The effect on the sense of orientation in light of adding acid to culture fluid

TIME	HCl CONCENTRA-TION ON BASIS OF PURE WATER	SENSE OF ORIENTATION	ALKALINITY AS INDICATED BY NEUTRAL RED
10.55	n/1000	Negative	Strongly alkaline
10.56	n/500	Negative	Strongly alkaline
10.57	n/333	Negative	Strongly alkaline
10.58	n/250	Negative	Strongly alkaline
10.59	n/200	Negative	Strongly alkaline
11.00	n/166	Clearly but only slight-ly positive	Definitely alkaline
11.03	n/166	Negative	Definitely alkaline
11.04	n/143	Definitely positive	Definitely alkaline
11.07	n/143	Clearly negative	Definitely alkaline
11.09	n/125	More clearly positive	Definitely alkaline
11.25	n/125	Definitely negative	Definitely alkaline
11.27	n/111	Definitely positive	Definitely alkaline
11.37	n/111	Slightly negative	Definitely alkaline
11.38	n/100	Positive	Definitely alkaline
11.42	n/90	Positive	Definitely alkaline
11.45	n/83	Positive	Definitely alkaline
2.00	n/83	Fairly strongly negative	Definitely alkaline
2.30	n/71	Definitely positive	Definitely alkaline
2.37	n/71	Definitely negative	Definitely alkaline
2.42	n/62	Definitely positive	Slightly alkaline
2.55	n/62	Definitely negative	
3.00	n/55	Definitely positive	Neutral?
December 13 a.m.	n/55	Definitely negative	Definitely alkaline
1.00 p.m.	Acid grad-ually add-ed	Definitely positive	Very slightly acid
1.30	No acid add-ed	Definitely negative	
2.00	Trace more acid added	Definitely positive	
2.10	No acid add-ed	Clearly negative	
24 hours later	No acid add-ed	Clearly negative	Very slightly alkaline or neutral
	Trace more acid added	All dead	

owing largely to the disappearance of the organisms in my cultures before sufficient tests were made.   Ten distinct tests were made on two different days.   In all of these tests the colonies were thoroughly washed in pure distilled water so as to remove all traces of the culture solution before the acid was added. The colonies were, under the conditions of the experiments, definitely negative in the pure water.   In seven there was no indication of reversion in orientation.   In one of the remaining tests some of the colonies probably became positive and in the other two a large majority of them clearly became positive. In these two tests the colonies had been in pure water overnight. They were tested in fresh pure water, but I am not certain as to whether or not they were washed in transferring them from the water in which they had been during the night to the fresh water. If pure water containing colonies is left in a watch-glass for some hours, it ordinarily becomes slightly alkaline.   It is therefore possible that the solution in which these colonies were tested was slightly alkaline, and it may be that this is the reason why reversion was obtained in these tests and not in the others.   It is consequently fairly certain that the reversion from negative to positive orientation in Spondylomorum is not due to the action on the organisms of acid or free hydrogen ions, as is maintained by some investigators.

If this is true, reversion must be associated either with certain chemical compounds produced by the action of the acids, as, for example, salts, or with the concentration of the hydroxyl ions. There is no evidence at hand in favor of the former supposition, as will be demonstrated presently, but there is a certain amount of evidence in favor of the idea that reversion is associated with the concentration of hydroxyl ions.   This evidence we shall now present.   Under certain conditions, changes in the alkalinity of the culture solution produced marked effects on the sense of orientation; under others it did not.   We shall discuss the latter first.

EFFECT OF ALKALIS

Experiments on the effect of changing the alkalinity were made at eight different times by adding sodium hydrate directly to the culture fluid containing positive colonies. Reversion was not obtained in any of these experiments, with the possible exception of those in which the colonies had been made positive by the addition of acid, and in none of these were the results clear-cut and definite.

Dilution of the culture fluid with pure distilled water also failed to produce definite results. The effect of such dilution in various degrees was tested in experiments made at nine different times. In four of these series of experiments the addition of pure water had no observable effect on the sense of orientation. In five the colonies became slightly positive when the pure water was added. To begin with, the colonies used in these five experiments were not strongly negative, and they soon became negative again after the water had made them slightly positive, and now the addition of more pure water did not make them positive again; on the contrary, it appeared to make them more strongly negative. It is consequently evident that reduction of alkalinity due to the addition of pure water was far less marked in its effect on reversion in orientation than reduction produced by the addition of acids. The results obtained by mixing culture solutions differing in alkalinity, as described below, were, however, quite as marked as those obtained by adding acids.

A total of nine series of tests were made in studying the effect of such mixtures on the sense of orientation. In some the alkalinity was increased, in others it was decreased. The concentration of the alkaline in the solution used was changed either by slow evaporation or by adding sodium hydrate. The results obtained were definite in every test. They may be summarized as follows:

1. Negative colonies in a culture solution concentrated by evaporation become definitely positive if fresh culture solution is added.

2. Positive colonies in fresh culture solution become definitely negative if culture solution concentrated by evaporation is added.

3. Negative colonies in culture solution which has been made more alkaline by the addition of sodium hydrate, become definitely positive if fresh culture solution is added.

4. Positive colonies in fresh culture solution become definitely negative if culture solution containing a little sodium hydrate is added. Reversion is, however, not permanent under any of these conditions. The results obtained in one of the experiments described in detail below are typical.

Culture jars containing Spondylomorum were frequently left uncovered in the laboratory. In some of these, owing to evaporation, the culture solution became considerably concentrated, and tests with neutral red showed that they were definitely more strongly alkaline than were the solutions in the covered jars in which evaporation had been prevented. On Dec. 18 numerous colonies were taken from one of these exposed cultures with some of the relatively strongly alkaline fluid and exposed in a given illumination. They were found to be strongly negative. After having been exposed for five minutes in this solution they were carefully transferred, without changing the illumination, to an equal amount of solution taken from a covered jar, i.e., to a solution not so strongly alkaline as indicated by the neutral-red test. In this solution they were first momentarily negative, then they became strongly positive, and remained so for six minutes, when some of them began to swim away from the light. Three minutes later, practically all of them were strongly negative.

These results show clearly that a decrease in the concentration of the salts and alkalis in a culture solution causes negative colonies of Spondylomorum to become positive, that is, it has the same effect as addition of acids. Now addition of acids produces an increase in the salt contents and a decrease in alkalinity. It is consequently evident that if the effect of acid is due to its effect on compounds in the solution it is not due to the production of salts, but to the neutralization of alkalis. We thus have a considerable amount of evidence favoring the idea that reversion in the sense of orientation produced by the addition of acids is due to a change in the concentration of hydroxyl ions. But whatever the action of these ions may be in the process of rever-

sion, it is certain that reversion is not specifically associated with their concentration, for organisms were repeatedly observed at different times to be negative as well as positive in practically all concentrations in which they oriented at all. Thus while the organisms tend to be positive in relatively weak and negative in relatively strong alkaline solutions, in four different experiments they were actually observed to be negative in solutions which were clearly slightly acid. Moreover, as may be observed by referring to the detailed description given above, of the effect of acids on the sense of orientation, the colonies change from positive to negative orientation without any appreciable change in the chemical constitution of the environment. This clearly shows that the sense of orientation is not specifically dependent upon the hydroxyl ion-concentration. Reversion is, however, not wholly independent of concentration.

EFFECT OF CHEMICAL CONCENTRATION

If acid, as previously stated, is added to a solution containing negative colonies they become positive, but after a certain time they become negative again without any further change in the solution. The time required for the reversion from positive to negative orientation depends upon the amount of acid added. For example, in one experiment, with sufficient hydrochloric acid added to make the culture fluid n/10,000 HCl, on the basis of pure water, it required approximately 3 minutes; with sufficient added to make it n/500 HCl, it required 3 minutes; with sufficient to make it n/333 HCl, it required 5 minutes; with sufficient to make it n/250 HCl, it required 6 minutes; with sufficient to make it n/200 HCl, it required a little over 8 minutes, and with sufficient to make it n/167 HCl, it required 84 minutes. In another experiment with sufficient hydrochloric acid added to make the culture solution n/1000 HCl, the colonies did not become positive at all; with sufficient added to make the solution n/200 HCl, they become definitely positive, and it required 6 minutes to become negative; with enough acid added to make n/143 HCl, they remained strongly positive for nearly one hour and then became neutral; whether or not they became negative later was not

ascertained. The solution gave a definite acid reaction with neutral red. The following day all of the colonies were dead and the solution was still distinctly acid. The fact that the time required for positive individuals to become negative is greatly extended when the proper amount of acid is added, seems to indicate that under certain conditions the colonies may be permanently positive. I was, however, unable to obtain such conditions.

While it has thus been clearly demonstrated that concentration is a factor in reversion, the following experimental results demonstrate equally clearly that the time rate of change in the concentration of the effective elements in the solution is also a factor.

EFFECT OF TIME-RATE OF CHANGE IN CHEMICAL CONCENTRATION

On December 5 numerous colonies in 1 cc. of solution were taken from an old culture from which considerable water had evaporated and exposed in diffuse light. The colonies were strongly negative. Hydrochloric acid was now added in sufficient quantity to make the solution n/1000 HCl on the basis of pure water. The HCl was now increased step by step through the following concentrations: n/500, n/333, n/250, n/200, n/166, n/142, n/125, n/111, n/100. During all this addition of acid there was at no time any indication of a reversal to positive reactions. The colonies were continuously definitely negative. But when colonies were taken from the same jar and put directly into the n/200 solution or into any of the solutions above this, they became strongly positive, remained so for several minutes, and then became negative again.

These results show clearly that the effect on reversion in orientation in Spondylomorum of slowly adding a certain amount of acid to a given amount of culture fluid is very different from the effect produced by rapidly adding the same relative amount of acid. We have previously demonstrated that the effect of acid on reversion is in all probability associated with the accompanying reduction in hydroxyl ions. If this is true, it may be concluded from the results now under consideration that it is dependent upon the time-rate of change in the concentration of the hydroxyl ions.

Whatever the processes within the organism may be that are induced by the environment in the production of changes in the sense of orientation, the same processes may, at least in part, occur without any immediate action of the environment as the following evidence shows.

EFFECT OF CHANGE IN PHYSIOLOGICAL STATES

As previously set forth, if the proper amount of acid is added to a culture fluid in which Spondylomorum is negative, it becomes positive, but ordinarily it remains positive only a few minutes and then becomes negative again.   If now fresh negative specimens from the same culture jar are added to the solution containing the acid, they respond just as the first specimens did when the acid was added.   They may be momentarily negative, but they soon become positive and remain so for a few minutes, after which they become negative and remain so.   This is clearly seen in the experiment described on page 508.

It was observed repeatedly in other experiments.   This demonstrates conclusively that the reversion from positive to negative orientation is not due to a change in the solution, but to a change within the organism, for if it were due to a change in the solution, the second lot of individuals, when added, should have responded just as the first set was responding at that time.

EFFECT OF ANESTHETICS

As previously stated, the addition of chloroform, ether, or chloral hydrate like the addition of acids causes negative colonies of spondylomorum to become positive, and the reactions of the colonies to these substances in reference to orientation are in detail essentially the same as their reactions to acids.   If a trace of chloroform, for example, is added to a culture solution containing negative colonies, they become, in the course of a few moments, strongly positive.   Then after a few minutes they become negative again.   If now more chloroform is added, they again become positive and after a few minutes again negative, etc., until the chloroform becomes sufficiently concentrated to produce anesthesia.   The reversion from positive to negative orientation is

not due to evaporation of chloroform, for if, after this reversion has occurred, fresh negative specimens are added to the solution, they become positive at once and then after a few minutes negative. That is, they respond just like the original colonies did immediately after the chloroform was first added.

The results obtained with chloroform and ether were quite as definite and conclusive as were those obtained with acids, but those obtained with chloral hydrate were much less definite.

In reference to the acids, it was concluded that their action on orientation. is probably associated with the time-rate of change in the concentration of the hydroxyl ions in the culture solution. The action of the chloral hydrate may have been due to the same cause, for it was slightly acid in reaction, but the action of the chloroform and ether could not have been due to this for both gave distinct alkaline reactions with neutral red. Moreover, it was found that chloroform produces reversion in negative colonies in chemically pure water, i.e., in a solution practically devoid of free hydroxyl ions.

EFFECT OF TEMPERATURE AND ILLUMINATION

The effect of illumination and temperature on the sense of orientation was not intensively studied. But the observations made indicate that strong light tends to induce negative, and weak light positive orientation, and they demonstrate clearly that an increase in temperature tends to make Spondylomorum positive and a decrease tends to make it negative. The relation between temperature or illumination and the sense of orientation is, however, not specific. For example, under certain conditions, colonies were found to be positive in all temperatures above 14° and negative in all below about 10°, while under other conditions the same colonies were found to be negative up to nearly 42° and positive above this temperature. Similar results were repeatedly obtained. Moreover, under certain conditions, reversion in orientation could not be induced by changing the temperature, and under other conditions it was observed without any change in temperature. Changes from positive to negative orientation in constant temperature are particularly prevalent

after the opposite reversion has been induced by an increase in temperature. Similar results were obtained in observations on the effect of light. Thus, it is evident, that the temperature or the illumination required to produce reversion in orientation in given colonies varies greatly. It should be emphasized here that the effect of light and temperature are opposite, i.e., an increase in temperature produces the same effect as a decrease in light and vice versa.

<div align="center">DISCUSSION</div>

The experimental results presented in the preceding pages indicate that reduction in alkalinity, increase in anesthetics, increase in temperature, and decrease in illumination, all have the same effect on the sense of orientation in Spondylomorum. They also indicate that the same change in the sense of orientation may occur without any appreciable change in the environment. It is consequently probable that reversion in orientation is due to some specific change in the physiological processes of the organism which can be induced by changes in any one of the environmental factors mentioned, i.e., alkalis, anesthetics, temperature, and light. What these processes are is not known. They may involve electrical tension and polarization or permeability or absorption.

We have demonstrated that reversion depends upon the time-rate of change of effective factors in the environment. This, however, does not prove that reversion is dependent upon the time-rate of change in the physiological processes involved rather than upon the state of the physiological processes as such, for we have also demonstrated that reversion induced by a given environmental condition is rarely, if ever, permanent. That is, if negative colonies are put into a solution containing the proper amount of chloroform, they become positive, but they usually remain positive only a few minutes and then become negative again. This indicates that there is rapid adjustment on the part of the organism to the new environmental condition. The fact, therefore, that reversion is not induced if the effective factors in the environment are slowly changed, may be due to adjustment

proceeding at the same rate as the environmental changes and not to lack of sufficient speed in the rate of change of physiological processes.

SUMMARY

1. Spondylomorum orients fairly accurately in light. It is negative under certain conditions and positive under others.

2. Chloroform, ether, chloral hydrate, all of the acids tested (carbonic, hydrochloric, nitric, sulfuric, formic, boric, chromic, tannic, tartaric, oxalic) when added to the culture solution cause negative specimens to become strongly positive. They have no effect on positive specimens except perhaps to make them more strongly positive.

3. Ethyl alcohol, ammonium chlorid, and pure water have no appreciable effect on positive colonies, but they probably cause negative colonies to become slightly positive.

4. Formalin, sugar, oxygen, hydrogen peroxid, magnesium sulfate, calcium chlorid, potassium nitrate, and all of the alkalis tested (sodium, potassium, and ammonium hydrate) have no appreciable effect on the sense of orientation.

5. Increase in the concentration of the culture solution produced by adding culture solution part of which has evaporated or to which sodium hydrate has been added causes positive colonies to become strongly negative. Decrease in concentration produced by adding a less concentrated culture solution causes negative colonies to become strongly positive.

6. Increase in temperature and decrease in illumination cause negative colonies to become positive. Decrease in temperature and increase in illumination cause positive colonies to become negative.

7. The sense of orientation is not specifically related to the concentration of chemicals in the environment. Spondylomorum probably may be either positive or negative in any solution in which it orients at all.

8. The effect of acids on the sense of orientation is probably due to the reduction of hydroxyl ions produced in the culture solution by the acids.

9. Reduction in the concentration of hydroxyl ions, increase in anesthetics, increase in temperature, and decrease in light, all produce the same reversion in the sense of orientation, and this reversion may also occur without any change in the environment. It is, therefore, probably due to some specific change in the physiological process in the organism, which may be induced by a number of different factors.

10. Reversion depends upon the time-rate of change in the concentration or intensity of the effective factors in the environment, but it has not been demonstrated that it depends upon the time-rate of change in the physiological processes which are involved in reversion.

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[2] For a fairly complete list of references to the literature on reversal in the sense of orientation, see Washburn ('17, pp. 200–214), Holmes ('16, pp. 116–119), and Mast ('11, pp. 264–287).

RELATIVE EFFECTIVENESS OF FOOD, OXYGEN, AND OTHER SUBSTANCES IN CAUSING OR PREVENTING MALE-PRODUCTION IN HYDATINA[1]

A. FRANKLIN SHULL

Zoological Laboratory, University of Michigan

CONTENTS

INTRODUCTION

A paper on the effect of oxygen on the life cycle of the rotifer Hydatina senta by Shull and Ladoff ('16) left certain matters in doubt. Whereas water saturated with a mixture of air and oxygen which contained 40 per cent of oxygen caused an increase in the ratio of male-producers to female-producers. saturation with a mixture containing 60 per cent of oxygen was attended by practically no results. Two explanations seemed possible. Either different concentrations of oxygen had different effects or oxygen was incapable of increasing male-production when male-production was already high from other causes. The

experiment referred to above, in which 60 per.cent oxygen pro-
duced no effect, was performed at a time when the rotifers were
going through one of their well-known 'epidemics' of male-pro-
duction (Shull, '15 b), whereas the experiment with 40 per cent
oxygen came at a period when male-production in the control line
was low. No opportunity was found to test these two possible
explanations, owing to the loss of the stock cultures of rotifers,
and the experiments were published while this question was still
unanswered.

The important discovery by Whitney ('14) that rotifers fed
upon a green organism, Chlamydomonas, produced many more
male-producing offspring than those fed upon other material,
raised new questions. Whitney took no account of certain
initial chemical differences between the contrasted lines nor of
the fact that green organisms yield oxygen as a by-product of
photosynthesis. Shull and Ladoff pointed out that although
nutrition might on a priori grounds be expected to have the effect
which Whitney ascribed to it, the other obvious factors should
be eliminated before attributing the residual effect to nutrition.
It remained a question, therefore, how much of the increase in
male-production which Whitney observed upon feeding with green
organisms was due to food, how much to.oxygen or other factors.

In the experiments described in this paper an answer to these
questions is found. The initial chemical differences referred to
above have been eliminated. The effects of oxygen and green
organisms have been measured and compared. In addition, the
effects of these two agents, which increase male-production, are
compared with two of the principal agents known to decrease
male-production.

EXPERIMENTS

Effect of sixty per cent oxygen

To show whether saturation with a mixture of air and oxygen
of which 60 per cent was oxygen caused any change in the amount
of male-production, the following experiments were performed:

Experiment 1. On each of the days named in table 1 three
female rotifers were placed in each of two dishes. In one was

poured some spring water and scum from a manure solution was added for food. The water put into the other dish was first saturated, by continued agitation, with an air-oxygen mixture produced by removing half the volume of air and replacing it with oxygen. Such a mixture must have been composed of about 60 per cent oxygen and 40 per cent nitrogen. Manure scum was added for food, as in the control dish. The dish was then placed under a sealed bell jar, from which half the air was withdrawn by a filter pump and replaced with oxygen. The air-oxygen mixture under the bell jar must have consisted of about 60 per cent oxygen and about 40 per cent nitrogen.[2]

After twenty-four hours all the parents were removed from these dishes. The young hatching from the eggs laid in that period, experiment and control alike, were reared to maturit ‘ in untreated water with manure scum as food. Whatever differences they exhibit, therefore, are due to differences in the conditions which affected the mother or early developmental stages. Table 1 shows the results. The excess of male-production in the presence of oxygen is fairly marked.

Experiment 2. This experiment was designed to contrast the effects of 60 per cent and 40 per cent oxygen mixtures by two simultaneous tests. In the absence of a second suitable bell jar, one test was made with 60 per cent oxygen, the next test with 40 per cent oxygen. It was hoped that by this alternation an epidemic of male-production, if one occurred, would occur in the control of both series of tests. Since, however, it was not feasible to make one test each day, and several days usually elapsed between tests, that object was only partially attained. It happened that on the whole the line used for control produced more male-producers on the days when 60 per cent oxygen was employed than on the days on which the 40 per cent mixture was used. How much this circumstance vitiates the conclusion to be drawn from the experiment is not known.

[2]The method of procuring air-oxygen mixtures of a given composition is here stated again specifically, since Whitney seems to have misunderstood the expressions '60 per cent oxygen' and '40 per cent oxygen.'

TABLE 1

Showing the effect of saturation of the water with an air-oxygen mixture, of which 60 per cent was oxygen, upon the ratio of maleproducers ($\male \female$) to female-producers ($\female \female$) in the rotifer Hydatina senta

DATE	AIR		OXYGEN	
	Number of $\male \female$	Number of $\female \female$	Number of $\male \female$	Number of $\female \female$
February 26................	2	6	2	16
February 26................	1	16	2	12
February 27................	6	30	1	21
February 27................	9	27	15	14
February 29................	4	28	17	19
March 1...................	0	20	2	24
Total...................	22	127	39	106
Percentage of $\male \female$	14.8		26.9	

The method of carrying out the tests was precisely the same as described for experiment 1, except that in the 40 per cent oxygen tests the mixture used in saturating the water and the mixture under the bell jar were made up of three parts air and one part oxygen, instead of equal parts of the two as in the 60 per cent oxygen mixture.

Table 2 shows the results of the experiment. In both of the tests the oxygen mixture produced more male-producers than the control. If the absolute difference between experiment and control be taken as the measure of effectiveness of oxygen, the 60 per cent mixture appears to be a little more potent than the 40 per cent. If the ratio of male-production in the experiment to male-production in the control is the proper measure of effectiveness, then 40 per cent oxygen produced greater results than 60 per cent oxygen. In other experiments the impression has been gained that the relative difference between experiment and control is a better measure of the effect produced than is the absolute difference. In this experiment the ratio of the percentage of male-production in oxygen to the percentage of male-production in the control is 1.51 in the case of the 40 per cent oxygen, 1.41 in the case of the 60 per cent oxygen. The fourteen tests made are too few to use statistically to determine whether

TABLE 2

A comparison of the effectiveness of a 60 per cent oxygen mixture and a 40 per cent
oxygen mixture in increasing male-production in the rotifer Hydatina senta.
The tests covered the same period of three months, but were made on
alternating days

DATE	AIR		60 PER CENT OXYGEN		DATE	AIR		40 PER CENT OXYGEN	
	Number of ♂♂	Number of ♀♀	Number of ♂♂	Number of ♀♀		Number of ♂♂	Number of ♀♀	Number of ♂♂	Number of ♀♀
March 6........	7	24	10	20	March 11......	1	25	1	27
March 13........	0	22	4	38	March 15......	6	22	2	38
March 18........	0	29	0	31	March 20......	0	25	3	21
March 23........	0	1	2	6	March 27......	12	11	35	2
April 4........	3	17	8	16	April 7........	1	4	0	6
April 10........	0	16	2	20	April 11........	0	14	17	9
April 13........	14	18	18	7	April 14........	1	30	2	29
April 17........	21	22	38	23	April 20........	3	38	3	45
April 25........	3	20	10	25	April 28........	1	44	5	37
May 2..........	4	15	7	19	May 5........	17	23	12	17
May 9..........	1	11	3	15	May 12........	1	1	0	17
May 19..........	8	28	1	13	May 30........	0	10	0	23
June 4..........	0	9	0	24	June 5........	3	34	3	22
June 8..........	1	25	0	16	June 12........	0	6	0	19
Total..........	62	257	103	273		46	287	83	312
Percentage of ♂♀..........	19.4		27.4			13.8		21.0	

the difference between these two ratios is probably significant or
not.

Amount of oxygen dissolved from atmosphere and from Euglena

After repeated tests had shown that the increase of male-produc-
tion in rotifers reared in oxygenated water was no mere accident,
it became a pertinent question whether, and to what degree, the
increase of male-production among rotifers fed upon green organ-
isms is dependent upon the liberation of oxygen in photosynthe-
sis.   As a preliminary problem, it was necessary to discover how
nearly the amount of oxygen dissolved from the air-oxygen mix-
tures described in this paper and in that of Shull and Ladoff

equaled that dissolved in water in which green organisms were kept as food. A number of tests to determine this relation were made, some of them closely resembling the experiments, others differing more or less, but bearing directly on the oxygen content of treated water. The oxygen in solution was measured by the Winkler method.

In this paper all measurements of oxygen are given in number of cubic centimeters per liter of water. This quantity is computed from the tests by means of the formula

$$o = \frac{55.825 \text{ b n}}{\text{t v}}$$

in which 'b' is the number of cubic centimeters of potassium bichromate solution used in standardizing the sodium thiosulphate; 't' is the number of cubic centimeters of sodium thiosulphate required in titration against 'b' cc. of potassium bichromate; 'v' is the volume in cubic centimeters of the water sample tested; and 'n' is the number of cubic centimeters of the thiosulphate required for the water sample. In the tests here described 'b' was always 25, the other factors being variable.

Direct solution of oxygen.—In table 3 are described the tests to determine the amounts of oxygen dissolved in water when the water is agitated in contact with an atmosphere containing an excess of oxygen. Test No. 1, consisting of seven parts, was most nearly like one of the oxygen experiments, and was furthermore most complete. This test indicates that the water used in one of the rotifer experiments contained 6.81 cc. of oxygen per liter at the outset (a). After it was shaken with an atmosphere of which 40 per cent was oxygen, it contained 11.33 cc. of oxygen per liter (b). When the food (manure scum) was added, the water contained only 7.58 cc. of oxygen (c). After standing a day under a bell jar, in an atmosphere of which 40 per cent was oxygen, the oxygen content of the water had risen to 8.35 cc. per liter (d). Had the dish been kept in air instead of under a bell jar, the oxygen content would have fallen to 5.43 cc. per liter (e).

TABLE 3

Showing the effect of agitation of water in contact with an atmosphere containing an excess of oxygen in increasing the oxygen content of the water. The columns of figures were obtained by Winkler's method of determination of dissolved oxygen

TEST NUMBER	TREATMENT OF WATER	VARIABLES IN FORMULA FOR COMPUTATION (SEE TEXT)			NUMBER OF CUBIC CENTIMETERS OF OXYGEN PER LITER
		(n)	(t)	(v)	(o)
1–a	Untreated water, without food or anything else, tested at once for oxygen...	8.9	20.7	88	6.81
b	Water shaken with 40 per cent oxygen atmosphere, tested immediately for oxygen...	14.8	20.7	88	11.33
c	Shaken with 40 per cent oxygen atmosphere, manure scum added, immediately filtered and tested for oxygen....	9.9	20.7	88	7.58
d	Shaken with 40 per cent oxygen, manure scum added, put under bell jar in 40 per cent oxygen atmosphere for a day, then filtered and tested...	10.9	20.7	88	8.35
e	Shaken with 40 per cent oxygen, manure scum added, kept in air a day, filtered and tested...	7.1	20.7	88	5.43
f	Untreated water, manure scum added, then immediately filtered and tested...	7.6	20.7	88	5.82
g	Untreated water, manure scum added, kept in air a day, then filtered and tested...	5.4	20.7	88	4.13
2–a	Shaken with 60 per cent oxygen atmosphere and tested immediately...	14.50	20.8	55	17.67
b	Shaken with 60 per cent oxygen, poured into six watch-glasses, kept under bell jar in 60 per cent oxygen atmosphere one day, then tested...	9.95	20.8	55	12.13
c	Poured into six watch-glasses, set beside bell jar one day, then tested...	6.05	20.8	55	7.88
3–a	Untreated water in twelve watch-glasses, manure scum added, left twenty-four hours, then tested...	5.97	20.7	88	4.57
b	Shaken with 80 per cent oxygen atmosphere, then tested at once for oxygen..	29.25	20.7	88	22.40
c	Shaken with 80 per cent oxygen, put into twelve watch-glasses, manure scum added, kept under bell jar in 40 per cent oxygen twenty-four hours, then tested...	10.23	20.7	88	7.73
4–a	Untreated water tested for oxygen...	13.3	20.7	120	7.47
b	Shaken with pure oxygen and tested immediately for oxygen...	40.5	20.7	120	22.75
c	Shaken with pure oxygen, kept forty-eight hours in atmosphere of 60 per cent oxygen...	32.3	20.7	120	18.14

The control started with 6.81 cc. of oxygen per liter in the un-
treated water (a). Upon the addition of food (manure scum) the
oxygen content fell to 5.82 cc. per liter (f); while after standing
a day it further fell to 4.13 cc. per liter (g).

Assuming that the oxygen content of the water in both halves
of the experiment changed uniformly during the twenty-four
hours in which eggs were being laid, the mean oxygen content
of the oxygenated water was

$$\frac{7.58 \ + \ 8.35}{2} = 7.965 \text{ cc. per liter;}$$

while the mean oxygen content of the control dishes was

$$\frac{5.82 \ + \ 4.13}{2} = 4.975 \text{ cc. per liter.}$$

Absolutely, the difference between experiment and control was
about 3 cc. per liter. Relatively, the oxygenated water contained
60 per cent more oxygen than did the untreated water.

Test No. 2 (table 3) is less complete than the foregoing, but
indicates approximately the amount of oxygen involved in the
60 per cent oxygen experiments. No test of untreated water at
the beginning of the twenty-four hour period described was made
and no food was added. It is likely that under these circum-
stances the untreated water exposed to air would not lose oxygen,
but might gain it, so that the average content of such water dur-
ing the twenty-four hours would be not greater than 7.88 cc. per
liter (c). The water shaken with 60 per cent oxygen must have
averaged about 15 cc. per liter (a and b), or nearly double the
content of the untreated water. This test was not very relevant,
but since the comparison was to be made between the 40 per cent
oxygen experiments and those in which green food was used, a
more accurate test of the 60 per cent oxygen experiments was not
made.

Tests 3 and 4 have little bearing on the experiments, and are
recorded for whatever interest may attach to the solubility of
oxygen at higher pressures.

Oxygen derived from photosynthesis. Euglena was used in these tests. Cultures of a species that formed a sheet of green animals in a somewhat inactive, though not encysted, state along the sides of the aquarium were maintained. A considerable quantity of Euglena was usually available, and it was possible to make nearly as thorough a test as was made with manure scum. Table 4 states in tabular form the nature of the tests and their results.

Test No. 5, of six parts, gives the best idea of the Euglena experiments described in this paper. If the results of this test be applied to one of the Euglena-rotifer experiments, presently to be described, they indicate that the water used in the experiment contained 6.05 cc. of oxygen per liter at the outset (a). When Euglena was added as food, the water contained 6.58 cc. of oxygen per liter (b). After four hours in direct sunlight, it contained 9.27 cc. per liter (c), but on standing overnight the oxygen content fell to 7.35 cc. per liter (d).

In the control the water contained 6.05 cc. per liter at the outset (a); 5.51 cc. after manure scum was added (e), and 4.63 cc. per liter after the dish had stood twenty hours (f).

It should be remarked that probably neither the Euglena nor the manure scum used in test No. 5 was as abundant as in one of the rotifer experiments, though the difference could not have been great. Accepting these results as typical, it appears that the mean oxygen content in one of the Euglena cultures was

$$\frac{\left(\frac{6.58 + 9.27}{2} \times 4\right) + \left(\frac{9.27 + 7.35}{2} \times 16\right)}{20} = \frac{31.70 + 132.96}{20} =$$

8.233 cc. per liter while the mean oxygen content of the control was

$$\frac{5.51 + 4.63}{2} = 5.07 \text{ cc. per liter.}$$

Thus the absolute excess of oxygen in the Euglena dishes over the control was about 3.16 cc. per liter. Relatively the Euglena dishes contained about 62 per cent more oxygen than the manure-scum cultures. As was pointed out above, the quantity

TABLE 4

Showing the effect of Euglena in increasing the oxygen content of water, particularly as contrasted with water containing manure scum. The columns of figures are the results obtained by Winkler's method of determination of dissolved oxygen

TEST NUMBER	TREATMENT OF WATER	VARIABLES IN FORMULA FOR COMPUTATION (SEE TEXT)			NUMBER OF CUBIC CENTIMETERS OF OXYGEN PER LITER
		(n)	(t)	(v)	(o)
5–a	Spring water, without treatment, tested at once for oxygen....................	7.9	20.7	88	6.05
b	Spring water, Euglena added, then immediately filtered and tested for oxygen.	8.6	20.7	88	6.58
c	Spring water, Euglena added, kept in direct sunlight four hours, then filtered and tested...........................	12.1	20.7	88	9.27
d	Spring water, Euglena added, kept four hours in direct sunlight, sixteen hours in diffuse light and darkness, filtered and tested...........................	9.6	20.7	88	7.35
e	Spring water, manure scum added, then immediately filtered and tested.......	7.2	20.7	88	5.51
f	Spring water, manure scum added, left twenty hours, then filtered and tested..	5.4	20.7	88	4.63
6–a	Spring water in flat dish, Euglena added, kept cool in direct sunlight four and one half hours, then filtered and tested for oxygen...........................	8.8	21.5	88	6.49
b	Spring water in flat dish, manure scum added, kept in diffuse light four and one half hours, then filtered and tested.	3.0	21.5	88	2.21
7–a	Untreated water in ten watch-glasses, Euglena and a little manure solution without scum added, kept cool in direct sunlight five hours, then in diffuse light and darkness fourteen hours, filtered and tested....................	5.73	20.75	67	5.75
b	Untreated water in ten watch-glasses, manure scum and a little water from Euglena culture (but without Euglena) added, kept in diffuse light and darkness nineteen hours, then filtered and tested...........................	3.83	20.75	67	3.84

TABLE 4—*Continued*

TEST NUMBER	TREATMENT OF WATER	VARIABLES IN FORMULA FOR COMPUTATION (SEE TEXT)			NUMBER OF CUBIC CENTIMETERS OF OXYGEN PER LITER
		(n)	(t)	(v)	(o)
8–a	Spring water in bottle, Euglena and a little manure solution without scum added, bottle sealed without air bubble, agitated on clinostat for two hours in direct sunlight and twenty-two hours in diffuse light and darkness, filtered and tested....................	16.91	20.75	115	9.89
b	Spring water in bottle, manure scum and a little Euglena water (but without Euglena) added, bottle sealed without air bubble, agitated on clinostat two hours in direct sunlight and twenty-two hours in diffuse light and darkness, filtered and tested..................	4.1	20.75	115	2.39
9–a	Spring water in bottle, Euglena added, bottle sealed without air bubble, agitated on clinostat thirty-two hours in diffuse light and darkness, filtered and tested.............................	20.2	20.7	115	11.84
b	Spring water in bottle, manure scum added, bottle sealed without air bubble, agitated on clinostat thirty-two hours in diffuse light and darkness, filtered and tested...................	3.55	20.7	115	2.08

of Euglena used in the rotifer experiments was probably greater than in the oxygen tests just described, so that the excess of oxygen in the Euglena dishes in the experiments was probably somewhat more than 62 per cent.

The decrease of the oxygen content of the water over night, in the Euglena culture, may be in part due to other organisms, since my Euglena culture was not quite pure. In this respect the tests described here apply more correctly to the rotifer experiments of this paper than they do to Whitney's experiments. Probably, however, a greater part of this overnight decrease is

due to escape of oxygen into the air. Quiet water in contact with ordinary atmosphere will not, as a rule at least, retain as much as 9.27 cc. of oxygen per liter.

The remaining tests in table 4 (Nos. 6 to 9) are so unlike any of the rotifer experiments that it is unnecessary to explain them further than they are explained in the table. All of them contribute, however, to the proof that Euglena under various circumstances markedly increases the oxygen content of the water in which it lives.

Euglena versus oxygen and other substances as male-producing or male-repressing agents

Since the quantity of oxygen produced by Euglena and dissolved in the water is about the same as that dissolved directly from the atmosphere in the rotifer experiments, the effects of Euglena and of oxygen in increasing male-production may be directly compared. Any excess of male-production in the Euglena experiments over that in the oxygen experiments may therefore be attributed to another factor, probably the food.

The following experiments were designed to compare the effectiveness of Euglena with that of oxygen among the male-increasing agents, and with manure solution and creatin among the male-repressing agents.

Experiment 3. Euglena versus oxygen. In each of three dishes was placed the same number of rotifers, the number ranging from four to eight on different days. Two of the dishes were filled with spring water; to one of these Euglena was added as food, to the other manure scum. In the third dish was placed water that had first been saturated with an air-oxygen mixture composed of about 40 per cent of oxygen and 60 per cent of nitrogen. Manure scum was added to the water in the third dish, which was then set under a bell jar in an atmosphere of which 40 per cent was oxygen. The chemical composition of the medium in the three dishes at the beginning of the experiment was equalized by adding to the Euglena-fed culture as much manure solution (without scum) as was added to the other two dishes with the scum, and by adding to the manure-scum cultures as

much water (without Euglena) from the Euglena stock as was introduced into the first dish with Euglena. It was easy to obtain this water practically free of Euglena, since the Euglena was a quiescent form that produced an incrustation along the sides of the jar, while very few individuals were actively swimming.

The parent rotifers were removed from their dishes from twenty-four to forty hours later. All young rotifers hatching from eggs laid in that time were reared to maturity, in all three lots alike, in spring water with manure scum as food. The male-producers and female-producers are recorded in table 5.

TABLE 5

Comparison of the effects of Euglena and manure scum as food, and of oxygenated and untreated water, upon the proportion of male-producers in the rotifer Hydatina senta

DATE	EUGLENA AS FOOD IN UNTREATED WATER		MANURE SCUM IN OXYGENATED WATER		MANURE SCUM IN UNTREATED WATER	
	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
March 20............	13	43	1	22	2	37
March 22............	21	33	15	33	1	21
March 23............	14	18	0	22	0	21
March 26............	29	28	1	29	0	18
March 28............	3	27	5	9	0	1
March 29............	25	55	2	77	3	74
April 2.............	4	62	4	41	10	44
April 4.............	14	61	4	64	0	59
April 16.............	1	46	0	28	0	35
April 18.............	1	16	23	45	13	58
April 19.............	3	68	0	53	3	57
April 23.............	6	27	0	20	0	5
April 25.............	0	47	5	51	4	30
April 26.............	0	34	3	47	0	54
April 30.............	0	57	0	31	0	40
May 2.............	0	32	1	86	0	69
May 3.............	1	25	9	26	3	46
May 7.............	2	43	0	47	1	18
May 9.............	4	67	0	41	0	50
May 14.............	0	29	1	21	0	20
Totals.............	141	818	74	.793	40	757
Percentage of ♂♀.	14.7		8.5		5.0	

Comparison of the second and third divisions of the table indicates that oxygenation of the water increases the proportion of the male-producers by 3.5 per cent. The first and third divisions show that Euglena increases male-production by 9.7 per cent, of which 3.5 per cent is presumably attributable to the oxygen liberated in photosynthesis, leaving 6.2 per cent to be caused by some other factor, probably the food itself. The food, if this assumption is correct, is a little less than twice as effective as the oxygen.

Experiment 4. Euglena versus manure scum, spring water versus manure solution. The effects of the four agents named, in conjunction with one another and in opposition to one another, are here tested. On each of the dates named in table 6 four rotifers were placed in each of four dishes. In two of the dishes was placed manure solution, the food in one of these being Euglena, in the other manure scum. Into the other two dishes was put spring water, the food in one of them being Euglena, in the

TABLE 6

A comparison of the effects of Euglena and manure scum, and of spring water and manure solution, upon the proportion of male-producers in the rotifer Hydatina senta

DATE	EUGLENA AND MANURE SOLUTION		EUGLENA AND SPRING WATER		MANURE SCUM AND MANURE SOLUTION		MANURE SCUM AND SPRING WATER	
	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
February 10	4	14	5	14	0	10	0	23
February 15	11	10	13	15	0	24	3	24
February 21	16	20	16	16	0	11	9	8
February 26	7	10	7	12	1	15	5	21
February 28	5	41	10	41	2	27	3	30
March 1	1	33	5	29	4	23	2	31
March 7	0	9	10	4	0	0	0	13
March 12	5	47	29	23	0	16	12	26
March 14	5	33	1	32	0	38	8	37
March 16	0	34	2	14	8	16	0	25
Totals	54	251	98	200	15	180	42	238
Percentage of ♂♀	17.70		32.88		7.69		15.00	

other manure scum. The two dishes receiving manure scum as food received also a small amount of water from the Euglena stock (without Euglena), and the dish receiving Euglena as food in spring water received also a small amount of manure solution (without scum). This was done in order to equalize the initial chemical composition of the media in the four dishes, except as it was intentionally made different by manure solution and spring water. After from twenty-four to forty hours the rotifers were removed from their dishes. All offspring hatching from eggs laid in that time were reared to maturity, all four lots alike, in spring water with manure scum as food.

Comparison of the third and fourth divisions of table 6 shows that manure solution reduces the proportion of male-producers by 7.31 per cent, but this reduction is more than offset by feeding with Euglena, as in the first division of the table. That is, Euglena increases male-production more than manure solution reduces it. If, however, as indicated by experiment 4, over one-third of the total effect of Euglena is due to the oxygen liberated in photosynthesis, then Euglena as food is less powerful in increasing male-production than manure solution is in decreasing it. The maximum male-production was obtained, as was to be expected, by using both Euglena and spring water (second division of table 6). The minimum is obtained from manure scum and manure solution together. This minimum is approximately doubled by substituting either spring water for manure solution or Euglena for manure scum. Making both substitutions practically doubles the results of a single substitution.

Experiment 5. Oxygen versus creatin, Euglena versus creatin. On each of the dates in table 7, three to five rotifers were placed in each of four dishes, the number in each dish being the same on any one day. The four dishes were treated as follows: 1) One lot was immersed in a dilute solution of crude creatin, of the concentration indicated in table 7; and fed Euglena. 2) One dish was filled with a measured quantity of water which had first been saturated with an atmosphere of which 40 per cent was oxygen. Enough of a more concentrated creatin solution was added to produce the same final concentration as in the preceding dish.

TABLE 7

Showing the effects of oxygen, Euglena, and creatin upon the proportion of male-producers in a Nebraska line of the rotifer Hydatina senta

DATE	STRENGTH OF CREATIN SOLUTION IN PER CENT	EUGLENA AND CREATIN SOLUTION		MANURE SCUM AND OXYGEN-ATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED SPRING WATER	
		Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
May 16..........	0.01	0	18	0	23	0	26	0	26
May 17..........	0.02	0	21	0	2	0	17	0	9
May 21..........	0.01	4	14	0	29	0	25	0	21
May 23..........	0.01	0	19	6	24	1	32	0	35
May 24..........	0.02	18	14	0	13	1	21	0	19
May 28..........	0.02	17	24	0	19	0	41	2	42
May 30..........	0.03	0	10	3	43	4	27	9	43
May 31..........	0.025	9	30	0	40	4	55	3	63
June 4..........	0.02	4	17	3	21	3	47	10	61
Totals		52	167	12	214	13	291	24	319
Percentage of ♂♀		23.7		5.3		4.2		6.9	

Manure scum was added as food, and the dish was set under a bell jar in an atmosphere of which 40 per cent was oxygen. 3) A third dish was filled with the dilute creatin solution, manure scum was added as food, and the dish set in air. 4) The fourth dish, as general control, was filled with untreated spring water, manure scum was added as food, and the dish was set in air.

After from twenty-four to forty hours the rotifers were removed from the dishes. The offspring hatching from eggs laid in that period were reared to maturity in spring water, being fed with manure scum.

The rotifers used in this experiment were received from Prof. D. D. Whitney, who collected them at Lincoln, Nebraska.

Experiment 6. This is a duplication of experiment 5 in every respect, except that the rotifers used were part of a line received from Prof. A. L. Treadwell, who in turn obtained them from Prof. D. D. Whitney, then at Wesleyan University. They are presumably from the same New Jersey stock as was used in Dr. Whitney's former work. All the preceding experiments in this

paper, with the exception of experiment 5, were performed with these New Jersey rotifers.

Table 8 shows the results of experiment 6.

While the last three divisions of table 7 show differences of the kind expected, these differences are small. Comparisons based on the first three divisions of that table would indicate that Euglena is about 18 times as potent in increasing male-production as is oxygen. Or, deducting from the total effect of Euglena the 1.1 per cent which a comparison of the second and third divisions of the table would indicate was due to oxygen, the effect of the Euglena as food would be nearly 17 times as great as that of oxygen.

A different result, however, is found in table 8. The first three divisions of the table show that oxygenation increases the proportion of male-producers 5.1 per cent, Euglena 20.2 per cent. Deducting from the total effect of Euglena the fraction chargeable to oxygen alone (20.2 − 5.1 = 15.1), Euglena as food is nearly three times as effective as oxygen in increasing male-production.

TABLE 8

Showing the effects of oxygen, Euglena, and creatin upon the proportion of male-producers in a New Jersey line of the rotifer Hydatina senta

DATE	STRENGTH OF CREATIN SOLUTION IN PER CENT	EUGLENA AND CREATIN SOLUTION		MANURE SCUM AND OXYGEN-ATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED SPRING WATER	
		Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
June 6........	0.02	19	11	2	29	2	19	0	28
June 7........	0.02	3	2	5	44	5	33	8	27
June 11........	0.02	3	19	0	21	0	6	0	11
June 13........	0.02	2	22	6	15	1	29	0	12
June 14........	0.02	4	28	6	26	1	29	4	23
Totals		31	82	19	135	9	116	12	101
Percentage of ♂♀		27.4		12.3		7.2		10.6	

Response to oxygen in the Nebraska line

The failure of the Nebraska line in experiment 5 (table 7) to show any marked effect of oxygen is the only inharmonious result obtained in this study. It seemed possible that this line was not as sensitive to oxygen as the New Jersey line used in the other experiments. If this were the case, any other response to oxygen might prove less marked in the Nebraska line than in the New Jersey line. Such a response to oxygen in this species was found in earlier experiments. In a former paper (Shull, '15a) it was shown that races of rotifers might differ very markedly in the place of egg-laying. One race laid its eggs very largely on the bottom or sides of the dishes; another race chiefly attached to the surface film of the water. In connection with later oxygen experiments (Shull, '18), it was shown that placing the dishes in an atmosphere containing an excess of oxygen caused the eggs to be laid more largely on the bottom than when the dishes were kept in air. If the Nebraska line lacked responsiveness to oxygen, this lack might be evidenced by failure of oxygen to alter the place of egg-laying. This possibility gave rise to the following experiment.

Experiment 7. On each of the days named in table 9 several rotifers of the Nebraska line were placed in each of two dishes of water and fed manure scum. One dish was set under a bell jar in an atmosphere of which 60 per cent was oxygen, the other placed under a bell jar in ordinary air. After a period of from sixteen to eighteen hours, the eggs laid on the bottom and at the surface film in each of the dishes were counted. As a check upon the results, a similar test was simultaneously made upon the New Jersey line. Table 9 gives the results.

While the Nebraska line is here shown to be responsive to oxygen, the controls show that it normally laid more of its eggs at the bottom than did the New Jersey line. In view of this normal difference between the two lines, it is difficult to judge of the relative effect of oxygen upon them, and the discrepant results in experiments 5 and 6 probably remain unexplained.

TABLE 9

Showing the number of eggs laid at the surface film of water and at the bottom of the dish by two lines of rotifers, one from Nebraska, the other from New Jersey, when kept in air and in a 60 per cent oxygen atmosphere. The excess of oxygen causes more eggs to be laid at the bottom, but it is questionable which line is most affected this way, since their behavior is different under the same conditions

DATE	NEBRASKA LINE				NEW JERSEY LINE			
	Air		Oxygen		Air		Oxygen	
	Eggs laid at surface	Eggs laid at bottom	Eggs laid at surface	Eggs laid at bottom	Eggs laid at surface	Eggs laid at bottom	Eggs laid at surface	Eggs laid at bottom
December 8.............	5	25	0	22	16	12	2	29
December 11.............	28	23	1	44	28	16	3	22
December 13.............	4	24	0	28	1	5	1	3
December 20.............	6	24	0	13	32	26	0	25
Totals................	43	96	1	107	77	59	6	79
Percentage at surface...	30.9		0.9		56.6		7.0	

DISCUSSION

While this paper was in preparation an article by Whitney ('17) on a similar subject, the relative effectiveness of oxygen and food as sex determiners, was published. These two papers, however, in no sense duplicate, since they approach the problem from different angles and with different criteria of judgment.

A considerable part of Whitney's paper is devoted to experiments to show that green organisms as food increase the production of males in other rotifers than Hydatina. It is to be regretted that many of these experiments were performed with mass cultures without controls. If these rotifers are like Hydatina in producing males in 'epidemics,' the lack of controls is most unfortunate. It is well known to every student of Hydatina that, especially in lines that produce only a moderate proportion of males, these males often appear in well-defined 'waves,' and it has been shown that these epidemics not infrequently have a rather regular periodicity (Shull, '15b). If, in an experiment without control, a line which regularly produces numerous males once a month, the experimenter, ignorant of the interval

between periods of many males, should attempt to alter male-production by introducing something into the culture five or six days before the beginning of the period of males was due, he could use any one of a dozen agents and obtain the same result, namely, an increase of male-production a few days later.

Notwithstanding the lack of controls, Whitney's conclusion that the use of green organisms for food in these several species of rotifer increases male-production is probably correct; it is undoubtedly correct in the case of Hydatina, as Whitney ('14) satisfactorily showed in an earlier paper and as I have confirmed in my experiments. The chief question is how much of this effect of green organisms is due to nutritive differences, how much to other agents associated with the green organisms.

One of Whitney's experiments bears upon this point, but the results are not easily understood. That experiment is represented by his table 5 (Whitney, '17). Information on which one could base a judgment of the significance of this experiment is not given. The cultures were apparently of some size, all of them containing green organisms. These cultures had been in existence from five to thirty days, presumably in the sunlight (either direct or diffuse), before the beginning of the experiment. Some of them were then excluded from the sunlight, but for how long a time is not stated. If the duration of the darkness was not prolonged, it is to be expected that the average oxygen content would be appreciably greater than in a culture which had never been kept in the light. The experiments described in table 4 of this paper, test No. 5, indicate that oxygen accumulated in photosynthesis is only very gradually lost. It is not clear, then, that the oxygen content of the cultures in the darkness was as much lower than that of the sunlight cultures as Whitney supposed. It is almost certain that, unless they were excluded from the sunlight for a considerable period, these dark cultures contained appreciably more oxygen than a culture never kept in the sunlight would contain.

It is a pertinent question, therefore, whether the smaller amount of oxygen in the cultures in darkness was as effective as the larger quantity in the sunlight. Some of Whitney's cultures

in the darkness were aerated, others not, and he appears to have suspected that if oxygen were a male-producing agent, the more oxygen there was present the more males there should be. In the experiments described in this paper there is little difference, in its effect on male-production, between water saturated with a 40 per cent oxygen atmosphere and water saturated with a 60 per cent oxygen atmosphere, although it was shown that 60 per cent oxygen atmosphere left the oxygen content of the water very plainly greater. What difference 'there was indicated that the lower concentration of oxygen was the more effective. It cannot be accepted without question, therefore, that aeration of the water, in addition to the use of green food with its incidental oxygen, should further increase the male-production.

Disregarding the above objections to the experiments on which table 5 of Whitney's paper is based, we may examine the results of those experiments. Instead of comparing only selected parts of his table 5, the whole table may be included. Since in some of the cultures more mothers were used than in others, merely adding together the offspring of parents that were treated in the same manner would give to those cultures having many mothers undue weight. A more just method of combining like cultures is to average the percentage of male-producers in all cultures; each culture is then as weighty as the others, regardless of the number of parents used. By this method of combination, Whitney's table 5 is converted into the following:

	Per cent male-producets
Sunlight, with aeration	54.5
Darkness, with aeration	19.0
Darkness, without aeration	40.6

It is difficult to see what these results mean, though Whitney concludes from them that oxygen is not a male-producing agent. Just why one is to infer that the quantity of food is the cause of the differences shown, is equally obscure. The argument for oxygen is at least as good as the argument for food.

The remainder of Whitney's paper is devoted mainly to the effect of oxygen upon the protozoan and bacterial food supply of the rotifers in experiments like those of Shull and Ladoff. He

finds that the increased oxygen content of the water favors more
rapid multiplication of these food organisms, and infers that the
rotifers were therefore better fed. That there is a certain low
concentration of food at which an increase of available food would
mean an increase of nutrition is probable. If food were in greater
concentration than this, it is not clear that further increase in the
amount of food at hand would cause an increase in the amount
eaten. A man who sits down alone at a table with a hundred
pounds of steak and fifty pounds of potatoes eats no more than
if only fifty pounds of steak and twenty-five pounds of potatoes
are set before him.

If the above criticism is not valid, and it is after all merely
quantity of available food that determines male-production,
that fact should be very simply and easily discovered by controlled
experiments, involving large numbers of individuals, and the
next desirable step in the investigation is perfectly clear.

The criticisms of Whitney's methods and conclusions in the
foregoing paragraphs may seem to indicate a wider divergence
between his conclusions and mine than really exists. Certainly
the conclusions reached in this paper and in that of Shull and
Ladoff differ less from Whitney's conclusions than Whitney's
recent article would lead one to suppose. Thus when Whitney
('17, p. 114) writes "According to Shull and Ladoff, one would
expect many male-producing females to be produced in sunlight
and Chlamydomonas with the accompanying excess of oxygen,
but no male-producing females would be expected to be produced
in darkness and Chlamydomonas with no excess of oxygen,"
he overdraws the indictment somewhat. The quoted statement
could be correct only if Shull and Ladoff had denied that food
had *any* effect on male-production, and had asserted that *all*
of the increase of male-production obtained by Whitney was due
to oxygen instead of food. No such idea was even suggested.
Indeed, there are frequent passages in the paper of Shull and
Ladoff that indicate the reverse. Thus on page 138 it is stated
"our results may be interpreted as being largely in support of
Whitney's contention." Again, page 156, "part of the increased
male-production following the use of Chlamydomonas as food

in Whitney's cultures, was due to the oxygen liberated by the green flagellates as a by-product of photosynthesis." Likewise, page 157, "We suspect * * * Whitney's conclusion that the food conditions influence male-production is correct." All that was insisted upon by the authors of these passages was that (page 157) "all the factors obviously associated with Chlamydomonas in the cultures should be separately tested before any residue of influence is assigned to nutrition."

Two factors associated with Chlamydomonas in Whitney's earlier experiments were 1) an initial difference in the chemical content of the water and 2) dissolved oxygen produced during photosynthesis. In this paper the former factor has been eliminated and the second has been measured. After deducting the measured effect of the oxygen from the total effect of the green organism used for food, there is a residual effect which it seems fair to attribute to nutrition. According to the results of experiments described in the foregoing pages, this residual effect due to nutrition is at least several times as great as the effect of the accompanying oxygen.

SUMMARY

Repetition of the experiments with an excess of dissolved oxygen, in which the excess was obtained by saturating the water with an atmosphere of which 60 per cent was oxygen, showed that oxygen is a male-producing agent. The experiments of Shull and Ladoff had demonstrated that a smaller excess of oxygen (obtained from a 40 per cent oxygen atmosphere) increased male-production, but left in doubt the effectiveness of a 60 per cent atmosphere. Rather direct comparison of the two concentrations of oxygen, in this paper, shows that there is little difference between them. The lower concentration may, perhaps, be a little the more effective in inducing male-production.

The amount of oxygen dissolved in the water in these experiments has been measured by the Winkler method. The cultures which were first saturated with an atmosphere of which 40 per cent was oxygen and were then enclosed in a vessel in such an

atmosphere, contained on the average about 60 per cent more dissolved oxygen than did the untreated controls.

Cultures in which Euglena was used for food were also compared, relative to their oxygen content, with cultures in which manure scum was used as food. The Euglena cultures under the conditions of the experiments, contained on the average about 62 per cent more oxygen than the manure scum cultures. Presumably, therefore, when Euglena increases male-production in Hydatina, as much of that increase is due to oxygen as is directly produced by saturation with a 40 per cent oxygen atmosphere.

In experiments with Euglena as food, after deducting the increase in male-production presumably due to oxygen liberated, it was found that Euglena was two or three times as effective as the oxygen, and in one case many times as effective. The experiment in which Euglena appeared to be many times as effective as its liberated oxygen was performed upon a different line from that of the other experiments. This line was tested to discover whether it was responsive to oxygen in another way (laying eggs at surface film or bottom of dish). While it was plainly responsive to oxygen, there is some doubt whether it was as responsive as the other line used.

Euglena as a male-producing agent was compared with manure solution as a male-repressing agent. The repressing effect of manure solution was a little more than offset by the Euglena (including the effect of the oxygen liberated by the Euglena).

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CONTRIBUTION TO THE STUDY OF EPITHELIAL MOVEMENT. THE CORNEAL EPITHELIUM OF THE FROG IN TISSUE CULTURE

SHINICHI MATSUMOTO (KYOTO)

Osborn Zoological Laboratory, Yale University

NINE FIGURES

CONTENTS

The mechanism of the movement of epithelium, embryonic as well as adult, has been discussed by various observers, but still further investigation is needed by the use of the tissue-culture method, where direct observation is possible.

The experimental work reported in the present paper is concerned with a study of the movement of corneal epithelium, and especially with its behavior with reference to certain mechanical supports.

Several papers have already appeared which deal with the movement of epithelium of various kinds in vitro (Ruth, '11, Champy, '14, Loeb, '02, Osowski, '14, and others). The observation of frog skin in vitro has been described by Holmes ('14) and Uhlenhuth ('14) recently in detail. Previous to this, Harrison ('10) Carrel and Burrows ('11), Lambert and Hanes ('13), and others mentioned it briefly in their papers on tissue culture. Among the works which deal with the culture of the corneal epithelium, that of Oppel ('12), who made use of the cornea of certain warm-blooded animals in his investigations, demands special attention. Harde ('16) made brief mention of an active lateral spreading of the corneal epithelium in the culture of vaccinia with corneal tissue. However, the materials which were used by Oppel and Harde are evidently not very suitable for direct observation. For this purpose cornea of more simple structure is desirable. It must be added that a number of investigations on the problem of the wound healing of the cornea have been made, such as those of Peters ('85), Salzer ('11), Löwenstein ('13), and others, which must, of course, be taken into consideration.

I. BEHAVIOR OF EPITHELIUM CULTIVATED IN VITRO

The cornea of the adult frog (especially R. pipiens) was used. After thoroughly washing its whole surface with sterilized Ringer's solution by means of a pipette, the entire cornea was cut out with a razor and put into Ringer's solution (or serum), after which it was divided into small pieces with very sharp scissors so that the fragments showed sharp edges. Pieces cut radially were preferred.

The cultures were all made by the hanging-drop method (Harrison, '10), the technique of which need not be detailed here. The piece of cornea was taken from the Ringer's solution and dropped on the surface of the cover-glass; excess solution was removed and a drop of plasma (or serum) run over the fragment; autoplasma was used in most of the cultures. The cover-glass was then inverted upon a thin glass ring and sealed on with vaselin (Harrison, '14).

Though all precautions were taken to keep the cultures free from bacteria, it was sometimes necessary to throw away a whole series as a result of infection. This was due obviously to the difficulty of perfect sterilization of the tissue.

In the aggregate, more than 1800 cultures were made, and in nearly all of the experiments two kinds of culture medium (plasma and serum) were used. The following descriptions are the results of the study of about 1500 cultures which were free from faulty technique.
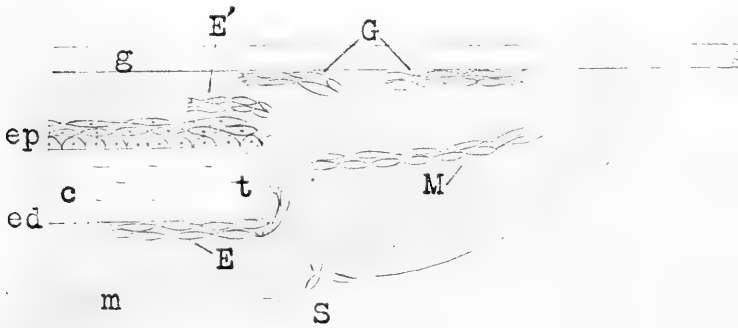
Fig. 1. Diagram showing various types of movement of corneal epithelium cultivated in plasma. *g*, cover-glass; *c*, tissue of cornea cultivated; *ep*, corneal epithelium; *ed*, endothelial surface; *m*, culture medium; *t*, cut end of the piece of cornea. Cell movement into the plasma (*M*), along the endothelial surface (*E*), on the epithelial surface (*E'*), along the cover-glass (*G*), and along the lower surface of plasma (*S*).

1. Description of the types of epithelial movement in plasma (fig. 1)

The cornea of the frog was very suitable for this purpose. For the observance of the intimate cell structure and a closer study of the mechanism of cell movement, high powers could be used. It was, of course, necessary to supplement the study of the living tissue by fixed and stained preparations of whole cultures and by serial sections. The observation of the living cultures was limited to the first week.

a. Movement in the medium. When a culture of cornea, freshly prepared in vitro, was examined under the microscope, the edges of the piece were seen to be sharply defined. One or two hours later the epithelium on the cut ends gave the impression of becoming a little translucent and swollen, a narrow clear rim appearing along the edges. Here and there, around the edges of the fragment, isolated round epithelial cells were to be seen, singly or in groups, having been detached by mechanical injury during the operation.

There was a short latent period before any movement was noticed, the first cellular activity appearing between the third and tenth hours. Accordingly, examination after twelve to twenty-four hours showed an active outgrowth of epithelial cells presenting amoeboid processes. As a rule, the corneal epithelium showed characteristic sheet-like extension during the period of active movement; the advancing edge was always furnished with an amoeboid border of hyaline ectoplasma, as has been described by Harrison ('10), Carrel and Burrows ('11), Lambert and Hanes ('13) and Holmes ('14). Some of the epithelium exhibited marked motility, recalling the movements of amoeba, so that an exact camera-lucida drawing could not be made; pseudopodia were formed, and through their activity the cells changed shape or moved from place to place. Some of the cells which showed filiform processes were over 0.2 mm. in length.

The strong tendency of the cells to lateral spreading brought about the formation of a continuous membrane, extending nearly horizontally, usually slanting upward a little toward the end. The spreading membrane also changed its direction of movement, showing contraction under various circumstances. Growth in strands was also observed. The growing epithelium, usually two cells in thickness, sometimes covered an area a little larger than the original corneal piece, whereby the cells became flattened and the intercellular spaces grew wider.

There was apparently little growth after the third (sometimes the fourth) day. The tissue itself then gave the impression of being less compact and more translucent than formerly. The epithelial cells showed a tendency to round off. The rupture

of strands or sheets of cells into isolated masses was of frequent occurrence. The number of round cells increased rapidly from day to day, and generally fat droplets grew in number and size with the age of the culture, until the cells were often literally packed with them. No marked increase of mitotic figures was seen.

The activity of cell movement into the plasma depends on the consistency of the latter. Around the explanted tissue, liquefaction and retraction of the plasma were often observed which caused changes of the arrangement of cells.
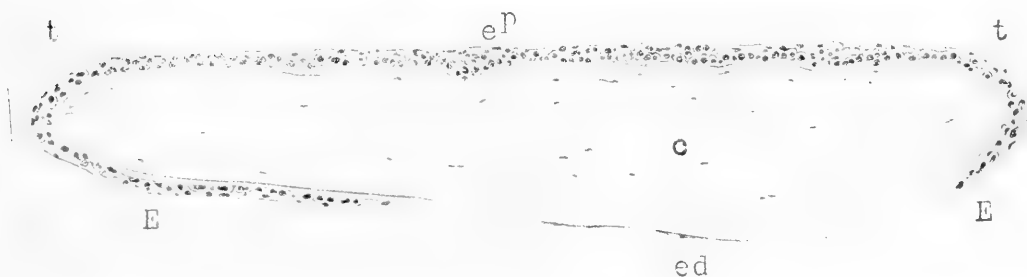
Fig. 2 Vertical section of corneal tissue cultivated in plasma. Experiment XXXVIII, 5, showing the epithelial movement (*E*) along the endothelial surface (*ed*). Age of the culture, four days. Drawn from one of the serial sections. *t*, cut ends; *ep*, epithelial, *ed*, endothelial surface; *c*, part of connective tissue of cornea. × 98.

b. Movement on tissue. The next important type of the epithelial movement is that on the corneal tissue. By virtue of this, the growing epithelium spreads over the edges of the fragment and along the endothelial surface. Figure 2 shows an example of this very clearly. A movement of this type occurred in some cases on all edges of a fragment and in others only on a part of it, combined with other types. It was also observed that part of the cut end of the epithelium might remain almost inactive, while in the other part marked activity occurred.

c. Other types of movement. Whenever the growing epithelial cells came into contact with the cover-glass, they moved actively over it. The character of cell movement, however, was essen-

tially the same as on the endothelial surface. The advancing
edge of the growth was composed of a very delicate sheet of
protoplasm, showing amoeboid movement, with numerous
branching hyaline pseudopodia. When the delicate membrane
spread out to an extreme degree, it resulted in the breaking up of
the sheet into isolated masses.

In a small percentage of the cultures the spreading of cells over
the outer epithelial surface of the cornea (fig. 1, E') as well as
along the lower surface of the culture medium (fig. 1, S) was
noted.

The elements of the connective tissue showed neither active
growth nor movement. The study of the lymphocytes found in
the corneal tissue needs special investigation.

2. Movement of the epithelium cultivated in serum

Generally, the cornea cultivated in serum showed some details
of cell movement, different from those noted in plasma cultures.
No amoeboid migration of the cells into the medium took place.
During the first and second hours, the cells of the edges became
clear and round, sometimes even swollen in appearance; they soon
started to move.

The most striking and constant phenomenon noted in the use
of serum was the spreading out of epithelium over the tissue
itself, especially on the endothelial surface. The epithelial rim
extended usually parallel with the cut edges, showing amoeboid
movement on the advancing border. The rapidity of spreading
out of cells was sometimes quite remarkable. In a small per-
centage of the cultures, movement of cells was also observed on
the original outer epithelial surface of the cornea.

If single cells or a part of the rim were in contact with the cover-
glass, they clung to it and spread over the surface of the glass with
marked activity. The boundaries of individual cells were often
hard to distinguish under the microscope. When such prepara-
tions are properly impregnated with silver nitrate (Lewis and
Lewis, '12) the intercellular spaces can be clearly demonstrated
(fig. 3). When the delicate membrane had extended to the

utmost degree, the cells along the periphery became loose and isolated.

The appended tables give some examples of the frequency of various types of epithelial movement in plasma (table 1) and in serum (table 2), respectively.
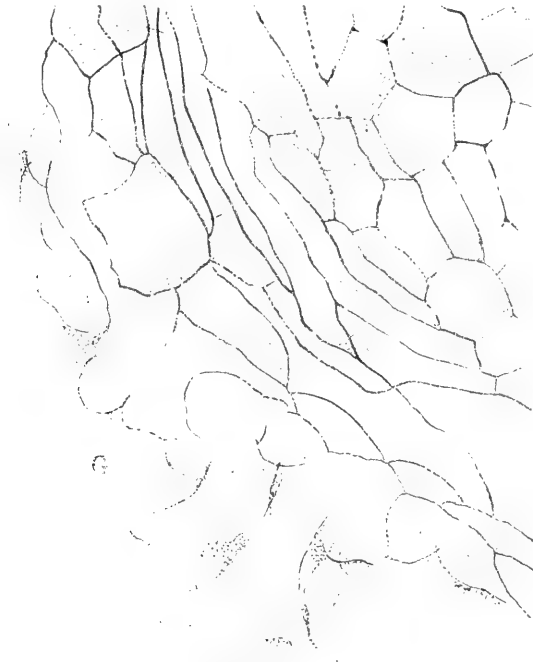
Fig. 3 Experiment XLIX, 2. Silver impregnation, demonstrating the boundaries of individual cells. Drawn from a part of actively moving border closely attached to the lower surface of cover-glass. Cultivated in serum; three days' growth. G, advancing border. × 450.

3. Velocity of epithelial movement

The velocity of cell movement in vitro exhibits a considerable variation under different circumstances, and, as stated before,

TABLE 1

Cultures in plasma

SERIES NO.	NUMBER OF PREPA-RATIONS	CELL MOVEMENT					NO MOVEMENT
		Into medium	On endothelial surface	On epithelial surface	On cover glass	On surface film	
50 a	18	14	14	1	1		
50 b	14	5	13				1
46	10	3	10		4		
45	6	5	5	2	2	1	1
43	20	13	11		2	1 (?)	
52	6	5	4	1	3		
57	10	9	3	2	4		
61	10	8	6	2	2	1	
62	2	2	2	2	2		
63	34	24	33				
102	33	18	31				
106	15	15	14				
104	15	1	15				
107	11	6	11				
110	8	6	8				
111	9	4	9				
212	18	11	17				
Total.....	239	149	206	10	20	3	2

combinations of several types of movement are often to be seen in a single preparation.

During the period of activity, a change in the type or direction of movement was often observed, and such a change in one part was apt to modify the movement of neighboring cells. Single cells or small groups of cells, as a rule, moved more freely than those in the spreading membrane. The consistency of the plasma, the liquefaction and the contraction of the fibrin influence both the rapidity and the type of movement into the plasma. The temperature has both direct and indirect influences on the movement. There is also individual variation which cannot be attributed to any of the above factors. For these reasons, the velocity of the epithelial movement varies from hour to hour, and it is very difficult to analyze the factors influencing it.

Usually, the most vigorous activity occurred during the first twenty-four hours. For instance, the epithelium on a piece of

TABLE 2

Cultures in serum

SERIES NO.	NUMBER OF PREPARATIONS	CELL MOVEMENT					NO MOVEMENT
		Into medium	On endothelial surface	On epithelial surface	On cover glass	On surface film	
56	28		28				
94	28		28		5	1	
58	7		6				1
60	8		7				1
139	7		7				
138	7		6				1
140	5		5				
135	8		8				
165	14		14				
166	12		12				
167	15		15				
208	23		23	1			
219	18		18				
221	20		20				
232	7		7				
233	9		9				
234	9		9				
Total.....	225		212	1	5	1	3

cornea cut radially into eight parts, spread out into the plasma within twenty-four hours in an extension which was even larger than their original area, though not in the same degree of rapidity at all cut ends. Similarly, in successful preparations, it was seen that the whole endothelial surface was covered within twenty-four hours by the epithelium spreading over the entire circumference of the piece. When the moving borders met each other, movement ceased.

Some measurements are given in table 3.

TABLE 3

	PREPA-RATION NO.	MOVEMENT ON ENDOTHELIAL SURFACE			MOVEMENT INTO PLASMA		
		After 5 hrs.	24 hrs.	48 hrs.	5 hrs.	24 hrs.	48 hrs.
Experiment 210, plasma culture, at 21°C.	1	0.14	0.4	0.5	0.06	0.3	0.5
	2	0.14	0.7	1.0	0.1	0.3	1.8
	3	0.1	0.8	0.8	0.11	1.1	1.6
	4	0.14	0.65	0.7	?	1.0	0.9
	5	0.14	1.0	1.0	0.08	0.8	1.0
	6	0.1	0.3	0.9			
	7	0.1	0.61	0.9			
	8	0.1	0.6	0.8			
Average movement in millimeters......................		0.12	0.63	0.83	0.087	0 7	1.16
					20 hrs.	40 hrs.	60 hrs.
Experiment 211A, plasma culture, at 20°C.	1				0.5	2 2	2.0
	2				0.8	4.0	5.4
	3				1.0	2.5	2.8
Average movement in millimeters......................					0.77	2.8	3.4
		20 h rs.	40 hrs.		20 hrs.		
Experiment 211B, plasma culture, at 20°C.	1	0.7	1.2				
	2	0.6	0.7				
	3	1.0	1.0				
	4	0.6	0 7				
	5	0.4	0.5		0.2		
	6	0.6	0.6				
Average movement in millimeters......................		0.65	0.78				
		20 hrs.					
Experiment 208, serum culture, at 20°C.*	1	0.3					
	2	0.4					
	3	0.8					
	4	0.8					
	5	0.6					
	6	0.7					
Average movement in millimeters......................		0.6					

*Pieces which measured: 1.2 x 0.6 mm.; 1.8 x 0.5 mm., 2.0 x 0.6 mm., 2.4 x 1.1 mm., and 0.8 x 0.8 mm., were entirely covered up with moving epithelium in seventeen hours.

II. ON THE REACTION OF EPITHELIAL CELLS TO CERTAIN SOLID SUPPORTS

1. Movement on flat surfaces

a. On glass and celloidin. We shall first consider movement on the glass cover-slip.[1] There is difficulty by means of usual culture method in bringing the epithelial rim into contact with the cover-glass from the beginning. Attempts to do this by reducing the plasma failed, as the epithelium did not show activity unless the medium was used in sufficient quantities.

In the later experiments, the piece of cornea (cut off tangentially to eyeball) was placed on the cover-slip, mainly with the inner surface down, and subjected to slight pressure by means of thin silver wires or glasses and kept in that position for a certain period after mounting with serum, so that the cut ends came into contact with the glass. When the silver wires were properly placed and controlled under the microscope, it was possible in almost every instance to bring certain parts of the moving epithelium into contact with the cover. Of course, where even a minimal space existed between the tissue and cover-glass surface, the epithelium crept on the underlying tissue.

Cover-slips coated with celloidin were also used. Thus the movement of the epithelium on celloidin and glass surfaces, respectively, could be compared. A comparison could also be made in the same preparation, by employing a cover-slip, only one half of which was coated with celloidin. Such preparations must be handled carefully, otherwise a change of cell form readily occurs.

No characteristic difference whatever in the mode of movement on the two different surfaces could be noticed. If the epithelium grew out vigorously, closely attached to the cover-slip, each cell became flattened into an exceedingly thin layer. In some instances it was seen that the advancing border became very

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[1] It should be stated here that the cover-glasses used in these experiments were throughly cleaned and washed in the vapor of distilled water, as is usually done to remove any trace of alkali, in order to exclude any chemical influence from that source. (Ostwald-Ruther, Physiko-chemische Untersuchungen.)

irregular. This was most marked when active cells on the border, showing unusual protoplasmic activities, tore themselves loose from their connection. Similar conditions, however, were often to be noticed in the actively mobile epithelial layer growing out into firmly clotted plasma. In the epithelial movement on the endothelial surface, such a condition but rarely occurred; as a rule, the cells moved more uniformly, showing a smooth advancing border.

In other instances small pieces of cover-glass or celloidin membrane were placed on the endothelial surface and pressed against it, so that a certain portion or the whole of this surface, which is a favorable support for moving cells, was covered. In these cases the epithelium was also seen to move over the celloidin or glass, if they were suitably mounted and the cells were not injured. Where the cell movement on such artificial supports failed, examination proved that the placing of the membrane was not suitable; thus, by control of pressure, it was possible to direct the moving cells to these artificial surfaces, though this was not always an easy matter. At any rate, it is an established fact that the epithelium is able to creep on objects of such nature that chemical influences are excluded.

b. On dead cornea. If in a part of an explanted piece of cornea an epithelial defect existed, it was seen that the epithelial cells were able to cover the spot, spreading out from all edges, practically with the same rapidity as on the endothelial surface.

The same was true of a spot which had been killed by touching it with a heated needle. The cells injured by the latter procedure became round and detached, and the growing epithelium crept beneath them along the surface of the killed tissue.

Preparations were made of large strips of corneal tissue (about 2 x 4 mm.), across the center of which a sharply defined epithelial defect was produced, after which one half of the remaining corneal tissue was killed by means of a heated needle, making the tissue surface look opaque and wrinkled. In such preparations it was seen that the epithelial movement over the wound occurred uniformly, covering both the live and the dead tissue with the same rapidity.

Analogous experiments were made with pieces of cornea in which an epithelial defect existed at one extreme end and in which the tissue at the other end had been killed; similarly, the cell movement on a burnt wound was compared with a control preparation, in which the underlying tissue was left intact with simply an epithelial defect.

The use of tissue, vitally stained by neutral red[2] or nile blue, facilitated the observation of epithelial movement on such wound surfaces.

Analogous observations were made by using the surface of the cartilaginous layer of the sclerotic coat of the frog's eye. This tissue could readily be isolated from the other layers after boiling, and it was then thoroughly washed before using. It was translucent, showing characteristic convexity, which admirably fitted on the inner surface of the similarly curved cornea; even without any pressing, the two tissues would lie so closely together in the culture medium (serum) that the epithelium from the cut ends of cornea crept over the cartilage. It proved helpful, however, if the pieces were slightly pressed together.

Out of twenty-six experiments, movement of the epithelium over the cartilaginous plate took place in twenty-one; in three cases no movement was observed owing to injury of cells. An example of this is illustrated in figures. 4 and 5.

2. Movement of fiber-like supports

Next, the response of the epithelium to the various fiber-like supports were tested, such as spider web, silk fiber, glass wool, and asbestos.

a. *Spider web as support.* Experiments were made similar to those of Harrison ('14), using spider webs. In each preparation a sufficient quantity of serum was used as culture medium.

Figure 6 represents one of the preparations of the series; many cultures were found where the cells clung to the fibers. Out of thirty cultures twenty-four were positive, four doubtful, two infected.

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[2] S. Matsumoto. Demonstration of epithelial movement by the use of vital staining. This paper will appear in the next number of this journal.

b. Silk fiber as support. Other experiments were made, using silk fibers. In these a number of fibers of raw silk were stretched on the glass ring and cover-glass, imitating the experiment with the spider webs.

Out of twenty-nine cases there were fourteen that gave positive results, one being infected.
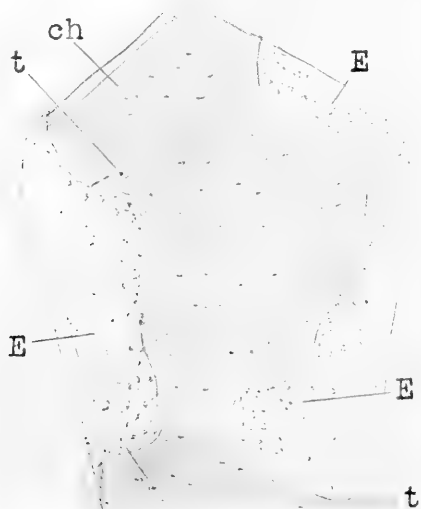
Fig. 4 Experiment 233, 4, showing epithelial movement (*E*) on the boiled cartilaginous plate (*ch*) of sclerotic coat closely placed on the endothelial surface of cornea. Cultivated in serum; forty hours' growth. *t*, cut ends of the piece of cornea are clearly visible through the transparent cartilagineous plate. × 98.

Similar experiments were made by Carrel and Burrows ('11a) with embryonic chick tissue.

c. Glass wool as support. Glass wool (Merck) was thoroughly cleaned and sterilized, then placed in the culture, so that the epithelium might attach itself to the fibers.

Out of twenty-eight cultures sixteen gave positive results. An example is shown in figure 7.

d. Asbestos fiber as support. Asbestos fibers were ignited and treated with pure conc. HCl; the fine cloudy suspension was collected and washed in distilled water to remove completely all traces of HCl, and then sterilized before use.

. In the serum culture of the cornea the fibers were mixed densely so that, they appeared like a nest, in which the tissue lay. As the fibers were very fine, they did not hinder microscopical examination, if not too densely placed.
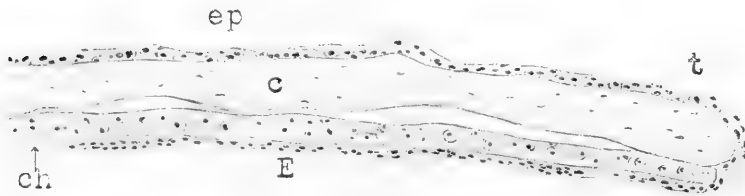
Fig. 5 Experiment 233; 4. Same preparation as shown in fig. 4. Portion of vertical section, drawn from one of the serial sections. *ep*, epithelial surface of cornea; *c*, connective tissue; *ch*, cartilaginous plate, placed on the endothelial surface of cornea; *t*, cut end of cornea. Note the epithelial movement (*E*) on the sclerotic cartilage (*ch*). Forty hours' growth. × 98.

Thirty cultures were made in this group, with positive results in twenty-two. Figure 8 shows an example of this series.

e. Movement on pith, etc. Further studies were made with pith. Thin pieces were used, which were kept in distilled water which was changed every three days for a period of over three months.

Experiments showed that the epithelium was able to cling to the cell walls of the thin piece of pith. Figure 9 shows clearly

the growing out of epithelial cells on the support.   Out of twenty-seven experiments nineteen gave positive results.

On the shell membrane of hen's egg, used as support, after thoroughly washing and boiling, a similar condition was observed.

6   7

Fig. 6   Experiment 238, 11.   Epithelial movement in serum on spider web. In the neighborhood of the explanted tissue (c) a good many isolated cells, round in shape, were noticed, which are omitted in this figure; they showed no active movement.   t, edge of the piece; above, some cells move out in sheets on the cover-glass.   Compare Jour. Exp. Zoöl., 17, 521, figs. 4 to 7, 12.   × 98.

Fig. 7   Experiment 49, 7.   Epithelial movement on glass wool (gw); culti-vated four days in serum.   t, edge of tissue.   × 98.

III. RÉSUMÉ

This paper deals with the movement of corneal epithelium of the adult frog in vitro.   Frog cornea is very suitable for this purpose, as it is so transparent and thin that it permits of direct observation of the cell movements.

The corneal epithelium cultivated in plasma shows various types of movement, according to the nature of the substratum

(fig. 1).   The movement is of an amoeboid character.   As a rule the cells have a strong tendency to cling to their own kind and thus extend in sheets, although under certain conditions active movement of isolated cells is also to be seen.

In the majority of cultures movement into the medium or along the endothelial surface (or both) takes place according to the consistency of the culture medium.   The fact, that the epi-

Fig. 8 Experiment 238, 1.   Epithelial movement on fibers of asbestos: cultivated forty hours in serum.   At the extreme left cells are to be seen moving along cover-slip.   Note the adaptation of single cells to asbestos fibers.   × 450.

thelium moves along the epithelial or endothelial surface is very important from various points of view; such a movement may easily be overlooked in the culture of non-transparent tissue, such as skin.

In the preparations in which serum is used, no migration of the epithelium into the medium takes place.   There is mainly a movement of cells over the tissue, especially on the endothelial surface.

Naturally, the question arises, whether the epithelial movement on the tissue, which is so frequently to be seen in the fluid medium, is chemotactic or thigmotactic in nature, It might even be that the type of movement is due both to mechanical and to chemical influences acting simultaneously. However, the epithelium is able to move with practically the same velocity both on a substratum where the covering epithelium has been simply
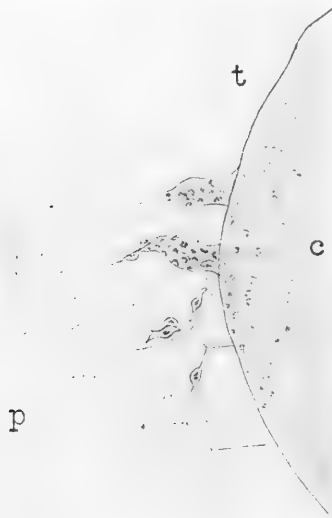
Fig. 9. Experiment 239. Corneal piece cultivated in serum with pith; three days after explanation. Note the epithelium clinging to the piece of pith (p) moving out of the cut end (t) of cornea (c). × 98.

scraped off and on one where the underlying tissue has been killed by heating. The movement in the latter cases cannot, therefore, be dependent upon chemotactic influences from the living tissue. The same is true of the movement taking place on the surface of the cartilaginous plate of the sclerotic coat, previously killed by boiling.

Furthermore, the epithelium is able to move on the surface of glass, on a celloidin film, and also on such fiber-like supports

as spider web, glass wool, asbestos, etc., when it is brought into contact with them.

The cell movement on the glass and celloidin film is very vigorous, sometimes more rapid than on the endothelial surface. In the movement on such supports chemotactic influences are to be considered as excluded.

It has been repeatedly observed by various writers that a suitable support for the growing cells is an important requisite; Harrison ('14) demonstrated lately the importance of such factors for the movement of embryonic cells very clearly. The facts above described confirm this view, that stereotropism plays an important rôle in cell movement.

The behavior of corneal epithelium in vitro serves to throw some light on the mechanism of epithelial growth in vivo.

The experiments show clearly that the epithelium is able to extend from the cut end quite rapidly in sheets into the medium (plasma), or on the tissue (plasma and serum), and can cover a large area, whereas mitotic cell divisions are not necessary at all.

That Oppel ('12) as well as Osowski ('14) did not observe amoeboid activity of the epithelium was perhaps due to the difficulty of direct observation. Special, careful observation on the movement of epithelium of warm-blooded animals, however, is necessary.

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